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VOLUME 1 | OCTOBER 1954 | NUMBER 2

MYCOBACTERIUM TUBERCULOSIS AND *CANDIDA ALBICANS*: A STUDY OF GROWTH-PROMOTING FACTORS[1]

By Edith Mankiewicz

Abstract

A new growth factor for *Mycobacterium tuberculosis* is described. It is produced by *Candida albicans* and stimulates the growth of tubercle bacilli of reduced viability or multiplication rate, as this is observed after treatment of the patient by chemotherapeutic or antibiotic agents. A method for the earlier detection of *M. tuberculosis* growing on Loewenstein's medium is described: Loewenstein's culture media, previously inoculated with the pathological specimen suspected to contain tubercle bacilli, are superinoculated with suspensions of *Candida albicans* whose dependence upon the presence of tubercle bacilli to grow on this medium has been enhanced. Colonies of *Candida albicans* will "trace" the presence of *M. tuberculosis.*

I. Introduction

Bacteriologists frequently see yeast cells in specimens of sputum submitted for examination for pathogenic bacteria. Most of these belong to the *Candida* group. Only in a few instances can the presence of these cells be related to mucosal lesions of the mouth or pharynx, or to lesions in the pulmonary parenchyma. An exceptional case which came under our observation recently revealed *Candida albicans* as a probable cause of certain pulmonary lesions.

Yeast cells are frequently found in the sputum of persons known to have pulmonary tuberculosis. This observation prompted the present study of a possible association between *Mycobacterium tuberculosis* and *Candida albicans.* To our knowledge, no similar studies have been made on this subject.

The strain of yeast cells selected for use in this work was the one isolated from the sputum of the above-mentioned case of pulmonary moniliasis. It was identified as *Candida albicans.** The pathogenicity of this strain was proved by the intraperitoneal inoculation into guinea pigs of 1 ml. of a 48-hr. culture in nutrient broth: at necropsy examination two weeks later, the animals presented multiple abscesses in the peritoneum and in the liver from which the fungus was recovered.

[1] *Manuscript received April 21, 1954.*
Contribution from Royal Edward Laurentian Hospital, Montreal, Que.

* *Dr. F. Blank, Department of Bacteriology, McGill University, to whom we extend our appreciation.*

[The first number of this Journal, (Can. J. Microbiol. 1:1-84. 1954) was issued August 1, 1954.]

II. Basic Studies

The Effect of M. tuberculosis on the Growth of C. albicans on Loewenstein's Medium

C. albicans grows readily on ordinary culture media, but poorly, if at all, on Loewenstein's culture medium for tubercle bacilli. We found that if tuberculin is added to this medium, by moistening the surface of the medium with a few drops of 1 : 10 or 1 : 100 diluted Old Tuberculin, the growth of *C. albicans* is promoted. However, instead of the large creamy white or yellow colonies observed on ordinary media, pin-point-sized and rather translucent colonies developed under these conditions. Microscopically, the predominant cell form in this case is the chlamydospore which develops mostly under unfavorable cultural conditions. If, instead of tuberculin, heat-killed tubercle bacilli are incorporated into Loewenstein's medium, *C. albicans* will develop creamy white colonies with round spores as predominant cells, such as are seen under favorable cultural conditions.

The fact that tubercle bacilli supplement the growth requirements of *C. albicans* in an otherwise deficient medium can also be demonstrated by the ability of these fungi to multiply in a saline suspension of heat-killed $H_{37}Rv$ or BCG organisms. Six serial transfers in such a "medium" confirmed the dependence of the fungus upon the presence of live or heat-killed tubercle bacilli for growth on Loewenstein's medium.

Each of two Petri dishes containing Loewenstein's medium was inoculated with two loopfuls of a 10-day culture of $H_{37}Rv$ in Dubos and Middlebrook's Tween medium. One Petri dish was kept in the incubator at 37° C. until, after 18 days, typical colonies of *M. tuberculosis* had appeared (Fig. 1). Six days after the inoculation of $H_{37}Rv$, when no macroscopic growth of *M. tuberculosis* was yet apparent, the other Petri dish was superinoculated with 3 ml. of a saline suspension of *C. albicans* grown on Sabouraud's dextrose agar after six transfers in saline-suspended tubercle bacilli. The excess fluid was removed and the Petri dish left in the incubator. After three days at 37° C., this plate showed creamy white colonies of *C. albicans* growing over the sites previously inoculated with tubercle bacilli. The rest of the plate showed no growth (Fig. 2). This basic experiment was repeated many times with identical results.

In order to explain this observation the bacteriostatic effect of malachite green was taken into consideration. Malachite green in concentrations of, and above, 0.0002% inhibits growth of *C. albicans*. The concentration of malachite green in the routine Loewenstein culture medium is 10 times as high as the minimum inhibiting concentration. Therefore, it would appear that colonies of *M. tuberculosis* provide a "foothold" free from malachite green for *Candida* cells which are not able to grow on the surrounding medium. This explanation, however, does not cover the growth-promoting effect of tuberculin, or of dead tubercle bacilli incorporated in the medium. It also does not take into account the appearance of *C. albicans* colonies *before* those of *M. tuberculosis* are evident.

PLATE I

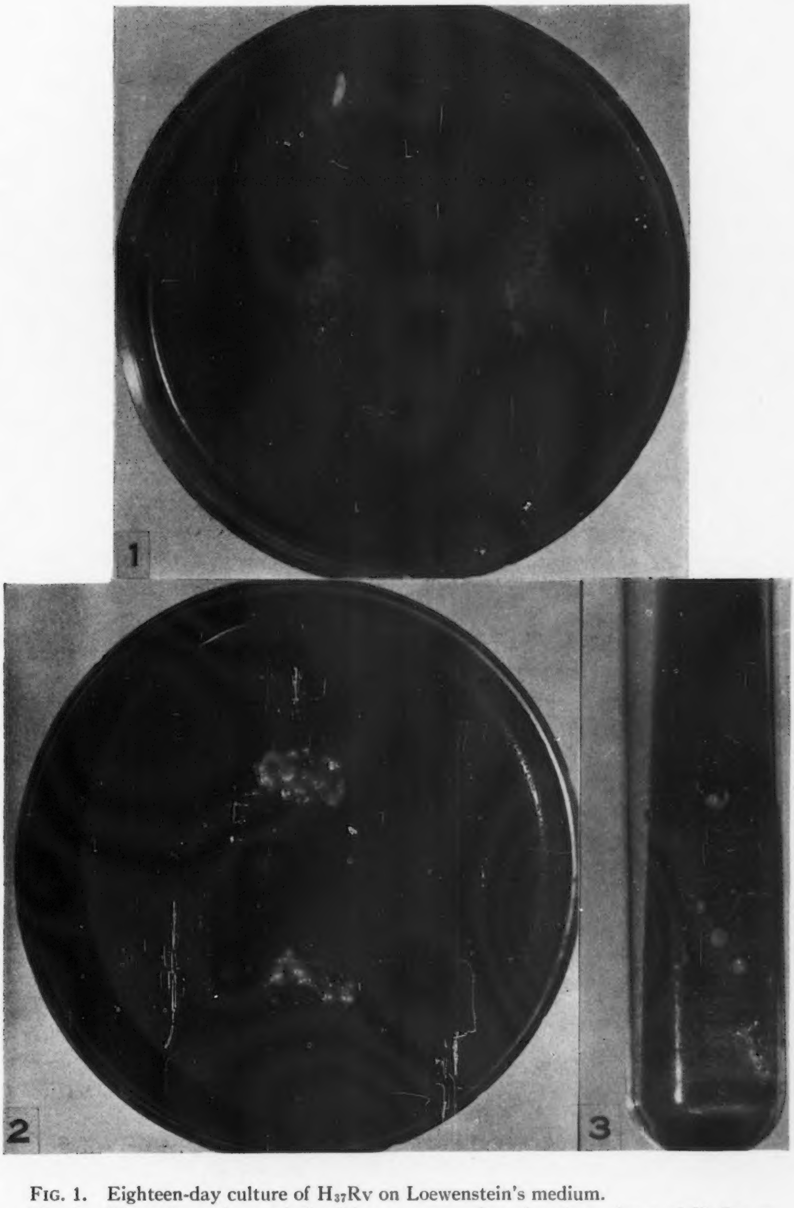

FIG. 1. Eighteen-day culture of $H_{37}Rv$ on Loewenstein's medium.

FIG. 2. Three-day culture of *C. albicans* overgrowing nine-day culture of $H_{37}Rv$ on Loewenstein's medium

FIG. 3. *C. albicans* colonies tracing those of *M. tuberculosis* in sputum culture on Loewenstein's medium.

C. albicans and the Tracing of M. tuberculosis in Sputum Cultures

For further study of this association, superinoculation with *C. albicans* suspensions of Loewenstein's media previously inoculated with *M. tuberculosis* was applied to the detection of tubercle bacilli in sputum or gastric lavage cultures of 200 clinic or hospital patients known to have, or suspected of having, active pulmonary tuberculosis. Concentrated sputum or gastric lavages from these patients were cultured in the usual manner on four Loewenstein culture tubes for each specimen. After 10 days' incubation at 37° C., when no growth of *M. tuberculosis* was yet visible, two of each of these tubes were superinoculated with *C. albicans*, whereas the other two were kept as controls. It should be noted that each set of cultures was made from the same concentrated specimen,—the control cultures first, and those destined to be superinoculated with *C. albicans* with the rest of the specimen which was, at times, poor material. This was done in order to reduce any possible sampling error which may have acted in favor of the superinoculation procedure.

In order to assure that no heat-killed tubercle bacilli were carried from the saline-suspended heat-killed tubercle bacilli "medium" onto Loewenstein's medium, three successive subcultures of *C. albicans* on Sabouraud's dextrose agar were carried out. Two loopfuls from the third 48-hr. subculture were suspended in 5 ml. of saline, and three drops of this suspension were used for superinoculating each Loewenstein culture. Three to twenty days later, smooth white colonies of *C. albicans* were observed on cultures of 42 out of 200 specimens (Fig. 3). The microscopic examination of smears from these colonies revealed the presence of acid fast bacilli among yeast cells of the predominantly round cell type. Five weeks after the inoculation of the specimens of sputum or gastric lavages, the control routine cultures were read. Thirty-one of the 200 specimens had produced macroscopic growth of *M. tuberculosis*. In 11 cultures the routine procedure had failed to produce growth of *M. tuberculosis*, while growth was promoted by superinoculation with *C. albicans*. In eight of these specimens, the direct microscopic examination had shown the presence of acid fast bacilli (Gaffky 2, 3, or 5).

Suspensions made from the above-mentioned 11 mixed cultures were treated with 3% sodium hydroxide for 20 min. at 38° C., then neutralized with 50% hydrochloric acid. When inoculated on Loewenstein's medium, they yielded pure cultures of *M. tuberculosis*.

Cultures of M. tuberculosis from Sputum of Patients Receiving Streptomycin or Isonicotinic Acid Hydrazide

One other interesting, and seemingly important, observation came out of the foregoing study. Six of the eight patients whose sputum contained acid fast bacilli which had failed to grow in the standard culture medium had been given—or actually were being given—streptomycin or isonicotinic acid hydrazide. This was taken as an indication that streptomycin- or isonicotinic acid hydrazide-inhibited tubercle bacilli may grow on Loewenstein's medium when superinoculated with *C. albicans*. To study this possibility, we used

the sputum of two hospital patients known to be positive for acid fast bacilli on direct examination (Gaffky 3) but where several attempts to culture these organisms on Loewenstein's medium had failed. One of the patients had received a total of 54 gm. of streptomycin in the interval between November 1953 and April 1954. The other patient had received a total of 137 gm. of streptomycin, 1926 gm. of PAS, and 96 gm. of isonicotinic acid hydrazide between November 1951 and September 1953. Since September 1953 this patient had continued the chemotherapeutic treatment with 300 mgm. daily of isonicotinic acid hydrazide.

Concentrated sputum of these patients was cultured once more on Loewenstein's medium, and the tubes were superinoculated with *C. albicans* in the manner just described. After a further 19 days, the typical smooth colonies of *C. albicans* were noted and, surrounding these, single rough colonies typical of *M. tuberculosis* had appeared. Microscopic examination showed that the latter were acid fast bacilli and that many such bacilli were also to be found among the yeast cells.

Conclusion

These observations point to a very strong dependence, at least of the "trained" strain of *C. albicans*, upon the presence of tubercle bacilli for growth of *C. albicans* on Loewenstein's culture medium. It is also noted that *C. albicans* contains, or produces, a factor promoting growth of tubercle bacilli. The effect of this growth-promoting factor extends to tubercle bacilli of reduced viability or multiplication rate which are now quite frequently found after prolonged treatment of the patient with streptomycin or isonicotinic acid hydrazide.

III. Practical Application

To make use of the growth-promoting factor produced by *C. albicans*, a new culture medium for tubercle bacilli was devised. Brain heart infusion broth (Difco) was inoculated with *C. albicans* and incubated at 37° C. After various intervals of time, from 18 hr. to seven days, cultures were filtered on paper and on Seitz. When the filtrates proved to be sterile they were distributed into tubes. These were inoculated with one of four strains of *M. tuberculosis*: the $H_{37}Rv$ strain, BCG, or with one of two virulent strains recently isolated from patients with pulmonary tuberculosis. It was noted on this occasion that the much-cultured laboratory strain $H_{37}Rv$ is able to grow on ordinary media, even on nutrient agar, and is therefore unsuitable for testing culture media.*

Growth of all four strains of *M. tuberculosis* was obtained consistently in the brain heart infusion broth in which *C. albicans* had previously been cultured. Most constant results were obtained with filtrates from 16- to

* *Noted by Dr. A. M. Masson, Department of Bacteriology, McGill University.*

24-hr. cultures of *C. albicans*, although occasionally filtrates from five-day cultures allowed heavy growth of tubercle bacilli. The growth is flake-like and deposits at the bottom of the tubes. No pellicle is formed.

The fact that this growth factor appears and disappears during the culture of the fungus suggests that it may be a metabolic product of *C. albicans*. Some stabilizing effect upon its presence in the medium was obtained by the addition of 2% gelatin to the brain heart infusion broth before its inoculation with the *C.* strain. Filtrates from cultures of *C. albicans* in gelatin brain heart infusion broth are now under study for their possible use as routine culture media for the isolation of *M. tuberculosis*.

EVIDENCE FOR MULTIPLE COMPONENTS IN MICROBIAL CELLULASES[1]

By William Gilligan and Elwyn T. Reese

Abstract

Evidence is offered for the existence of several cellulolytic components in the extracellular filtrates of microorganisms. Components separated chromatographically differ in their relative activities on native cotton, swollen cellulose, and CMC. A synergistic effect was obtained when separated components were recombined.

Introduction

During the last few years data have been accumulating which show that the natural polymers are hydrolyzed not by a single enzyme but by a number of enzymes acting on the same linkage in different ways. While it is true that two amylase types have long been known, the tendency has been to assume that the polysaccharase or protease of any particular organism is of a single type. Even microbial amylase was considered to be solely of the alpha amylase type until recently (3, 4). The multiplicity of enzymes acting on the same substrate has been demonstrated chiefly by chromatographic techniques. As examples, we may cite four active components in fungal amylase (17), two in polygalacturonase (17), two in yeast invertase (1), and two in fungal proteinase (2). That more was involved than mere differences in rate of movement on columns (or on paper) was shown by demonstration of differences in (*a*) rates of activity on various substrates, (*b*) temperature and pH stability, (*c*) specific rotation of the hydrolysis products (3, 4).

In the cellulolytic system of microorganisms, the detection of several components has been made by means of paper chromatography and paper electrophoresis. Jermyn (6) has shown that fractions obtained from *Aspergillus oryzae* differed in their relative activities on β-glucosides and on carboxymethyl cellulose (CMC). Reese and Levinson (16) found that the cellulases of different organisms differed in their relative activities on CMC and on cotton. On the other hand, Whitaker (20) maintains that only a single cellulolytic component is present in *Myrothecium verrucaria* filtrates. The present work describes the further separation of enzyme components and the nature of some of the differences between them.

Methods

Activity Measurements

$Cx\ \mu$ = amount of enzyme in 10 ml. of assay medium (0.5% CMC 50T in $M/20$ citrate, pH 5.4) required to give a reducing value (as glucose) of 0.40 mgm./ml./hr. at 50° C.

[1] *Manuscript received June 7, 1954.*

Contribution from Pioneering Research Laboratories, Philadelphia Quartermaster Depot, Philadelphia 45, Pa.

Walseth (W) μ = amount of enzyme in 2 ml. of assay medium (0.5% suspension Walseth cellulose in $M/20$ citrate, pH 5.4) required to give a reducing value (as glucose) of 0.50 mgm./ml. in two hours at 50° C.

The Walseth cellulose was prepared by swelling ground cotton in 85% phosphoric acid according to the procedure of Walseth (19), except that a prehydrolysis step with 1% hydrochloric acid (reflux for one hour) was employed.

Swelling factor (SF) μ = amount of enzyme per ml. of filtrate sufficient to obtain a swollen weight of the cotton fiber of 50 mgm. above the control value (in one hour at 50° C. pH 5.4) under the conditions of the alkali swelling test (Marsh *et al.* (9); Reese and Gilligan (14)).

The units obtained (above) are dilution units, i.e. the test solutions are diluted with $M/20$ citrate buffer pH 5.4 to the point where a certain rate results. The selection of the one-unit rate is such that it falls within the linear portion of the curve (activity vs. time; or activity vs. enzyme concentration). The use of these terms ($Cx\ \mu$, $W\ \mu$, $SF\ \mu$) is *not* meant to imply that a different enzyme is involved in each measurement. Each "cellulase" probably acts on all three substrates, differing however in the *relative* activity on each.

$R\ W/Cx$ is the ratio of Walseth units to Cx units. $R\ SF/Cx$ is the ratio of SF units to Cx units.

Preparation of Enzyme Concentrates

The fungi were grown in shake flasks on 0.4% cellulose in mineral salts solution (16). The development of the activity was followed and the cultures harvested when the Cx activity reached 10–15 μ/ml. for *M. verrucaria* QM 460 (5–10 days), and 12–16 μ/ml. for *Trichoderma viride* QM 6*a* (9–18 days). The mycelium was removed by filtration and discarded. The filtrate was treated with 2 (−3) volumes cold acetone, allowed to stand overnight in the refrigerator, and the precipitate was then removed, air-dried, and stored. The *T. viride* precipitate at this stage contained about 75% water soluble material which could be removed by dialysis. After dialysis the precipitate appeared to be about two-thirds protein. It is this material that was used in the chromatographic work. (*M. verrucaria* concentrates were prepared in the same way.)

Paper Chromatography

Sheets of zein-treated filter paper (1 × 40 cm.) were spotted six centimeters from one end with concentrated enzyme solution, and developed overnight with 0.3 M sodium chloride in $M/20$ citrate (pH 5.6, 8° C.). The paper was air-dried at room temperature and cut into sections 1 (−2) cm. long. These sections were assayed by incubating directly with the assay medium (Reese and Gilligan (13)).

In the next phase of our work, wider strips (15 × 40 cm.) were used and 1 ml. of enzyme concentrate added along a line 6 cm. from the top. The addition of this volume of solution must be done in several steps. After development, the strips are cut in 1 cm. lengths and the aliquots (1 × 15 cm.) eluted at 50° C. for 30 min. in 8 ml. $M/100$ citrate (pH 5.6).

Column Chromatography

In column experiments, cellulose was poured into the tube as a slurry at the desired pH. Some forms of cellulose (Walseth cellulose) require a filler (celite 545) to obtain a satisfactory flow rate. The enzyme solution was added to the top of the column, and eluted at pH 5.6. Aliquots were collected and assayed for activity. In some cases the column was extruded, cut into sections, and subjected to further elution. When reducing sugars were present in any quantity, the eluates were dialyzed prior to assay.

Calcium phosphate gel was prepared following the procedure of Swingle and Tiselius (18). For use in columns, one part of the gel was diluted with 10 parts of celite 545. The column was washed with water and with 2% sodium chloride prior to the addition of the dialized enzyme solution. Elution was achieved by successively increasing the concentration (0.002–0.02 M) of potassium phosphate buffer (pH 7.0) in 2% sodium chloride. For large columns (43 × 210 mm.) to which 300 mgm. of enzyme concentrate (dry wt.) was added, the eluates were of 90 ml. each, and the rate of flow (using 10–15 lb. air pressure) 10 ml./min. Activity assays were carried out on the eluates. The eluates of highest activities were dialyzed through animal membranes, and these solutions used in the characterization of components.

Results

(*A*) *Variation in Cellulolytic Properties of Filtrates*

Organisms were grown on duck strips in test tubes of mineral salts solution (16) and the culture solutions assayed for *W*, *SF*, and *Cx* activity. Comparison of the *R* values in one culture filtrate with those of another filtrate shows that there is wide variation among organisms in the relative amounts of the enzyme components. Those organisms showing the greatest differences have been selected for use in Table I. Thus we see that the *R W/SF* of a filtrate of *Helminthosporium* sp. is 90 times that of *Aspergillus luchuensis;* the *R W/Cx* of *Fusarium roseum* 20 times that of *Cellvibrio vulgaris;* and the *R SF/Cx* of *Fusarium moniliforme* 80 times that of *Streptomyces* sp.

The relative amounts of the enzyme components vary also for a single fungus growing under different conditions. This is quite marked in *M. verrucaria* (Fig. 1). As the amount of cellulose in the growth medium is increased, the Walseth activities and the swelling factor activities decrease rapidly relative to the *Cx* activity (Fig. 1*A*). At the highest cellulose concentration (3%, Fig. 1*B*), the *Cx* activity of the medium remains constant for some time after the maximum value has been reached, but the Walseth activity drops rapidly.

TABLE I

VARIATIONS IN RELATIVE ACTIVITIES OF CELLULOLYTIC SYSTEMS

Organism	R‡	Activity, μ/ml.* W	SF	Cx	Growth† conditions
R W/SF High					
Polyporus versicolor QM 1013	10+	7.2	0	5.0	T21
Helminthosporium sp. QM 1218	9	75.0	8.0	7.5	T21
Scopulariopsis brevicaulis QM 815	7	7.4	1.1	1.9	T21
Schizophyllum commune QM 812	5	12.0	2.2	6.8	T21
R W/SF Low					
Trichoderma viride QM 6a	0.12	16.0	128.0	8.0	F7
Aspergillus luchuensis QM 873	0.10	4.4	42.6	4.5	T21
Aspergillus terreus QM 72f	0.06	14.3	256.0	17.1	T21
R W/Cx High					
Helminthosporium sp. QM 1218	11.0	91.0	8.0	8.4	T24
Fusarium roseum QM 38g	10.0	83.0	120.0	8.0	T21
Stachybotrys atra QM 94d	10.0	36.0	66.0	3.6	T21
Fusarium moniliforme QM 1224	9.0	35.0		4.0	T24
R W/Cx Low					
Pestalotia palmarum QM 381	1.0	29.0	44.0	30.0	T90
Penicillium pusillum QM 137g	0.9	27.0	27.0	30.0	T74
Aspergillus terreus QM 72f	0.8	14.0	256.0	17.0	T21
Cellvibrio vulgaris QM B2	0.5	3.5	4.5	7.6	F13
Sporocytophaga myxococcoides QM B482	0.6	0.8	0.7	1.4	T13
R SF/Cx High					
Fusarium moniliforme QM 1224	24.0	17.0	56.0	2.3	T21
Stachybotrys atra QM 94d	18.0	36.0	66.0	3.6	T21
Trichoderma viride QM 6a	16.0	16.0	128.0	8.0	F7
Aspergillus terreus QM 72f	15.0	14.0	256.0	17.1	T21
Fusarium roseum QM 38g	15.0	82.5	120.0	8.0	T21
R SF/Cx Low					
Penicillium pusillum QM 137g	0.9		27.5	31.0	T74
Cellvibrio vulgaris QM B2	0.6	3.5	4.5	7.6	F13
Sporocytophaga myxococcoides QM B482	0.5	0.8	0.7	1.4	T13
Monotospora brevis QM 1243	0.4		4.2	10.4	T13
Streptomyces sp. QM B1555	0.3	14.0	6.7	23.3	T

* *All activity measurements at 50° C. pH 5.4 ± 0.1 and one hour (two hours for Walseth activity). W = activity on Walseth cellulose; SF = swelling factor; Cx = activity on CMC.*

† *Growth: T = test tube cultures; F = shake flasks; number = days of incubation.*

‡ *R refers to the ratios shown at the left of table.*

As a result the *R W/Cx* values decline with time of incubation to a low of 0.17 (the high value for filtrates of this organism being about 2.6–3.0). This change in *R W/Cx* is much less pronounced at lower cellulose concentrations.

We had found earlier that a cellulose concentration of about 1% is optimal for *Cx* production by *M. verrucaria*, for the type of cellulose (Solka Floc) that we are using. In our tests, however, we routinely employ a concentration of 0.4–0.5%, and as a result obtain filtrates having *R W/Cx* and *R SF/Cx* values of about 2.0 and 9.0 respectively (Fig. 1).

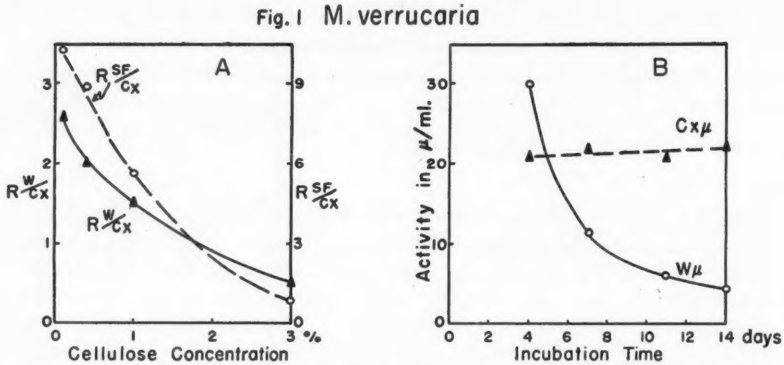

FIG. 1. Effect of cellulose concentration in the growth medium, and of time of incubation, on the nature of the cellulolytic enzymes of *Myrothecium verrucaria* filtrates. (Shake flask expt.). *A*. Comparison of filtrates of cultures eight days old. *B*. Changes in enzymic activity of filtrates of a culture grown on 3% cellulose.

(*B*) *Separation of Components*

(*1*) *Paper Strip Chromatograms*

When crude enzyme filtrates are chromatogrammed, several *Cx* components are frequently found (Reese and Gilligan (13)). When replicate strips are cut up and incubated with Walseth cellulose (instead of CMC) chromatograms are obtained which resemble the *Cx* chromatograms in the position of the peaks but differ in the activity at those peaks (Fig. 2*B*). The enzyme components, first identified by their rates of movement on paper strips, differ from each other in their relative activity on the two substrates used.

The method of assaying pieces of paper from the developed strip (above) does not readily permit quantitative evaluation. To characterize the components, solutions of each fraction are required so that activities may be obtained on a unit basis. For this purpose wider strips (15 cm.) were used, and heavy dosages of enzyme concentrate added. Eluates from the 1 × 15 cm. aliquots were assayed for both *Cx* and Walseth activities. From these figures a ratio of Walseth units to *Cx* units can be derived (*R W/Cx*).

There are some differences between chromatograms of strip eluates and chromatograms obtained by direct immersion of paper strips in the substrate solution. Some components are not eluted readily under the test conditions, and these may hydrolyze CMC even when they are adsorbed on the paper. That adsorption on the paper is an important factor is shown by the poor recovery of activity in the eluates (for *T. viride* 37% recovery of *Cx*; 16% recovery of *W*). Of greater importance is the ability to follow the relative activities of the fractions on the two substrates (*R W/Cx*). In filtrates of *M. verrucaria* three components are detectable (Fig. 2*A*; 20 hr. development):

Slow moving, low *R W/Cx* remaining at initial spot;
Slow moving, high *R W/Cx* moving slightly from initial spot;
Fast moving, low *R W/Cx*.

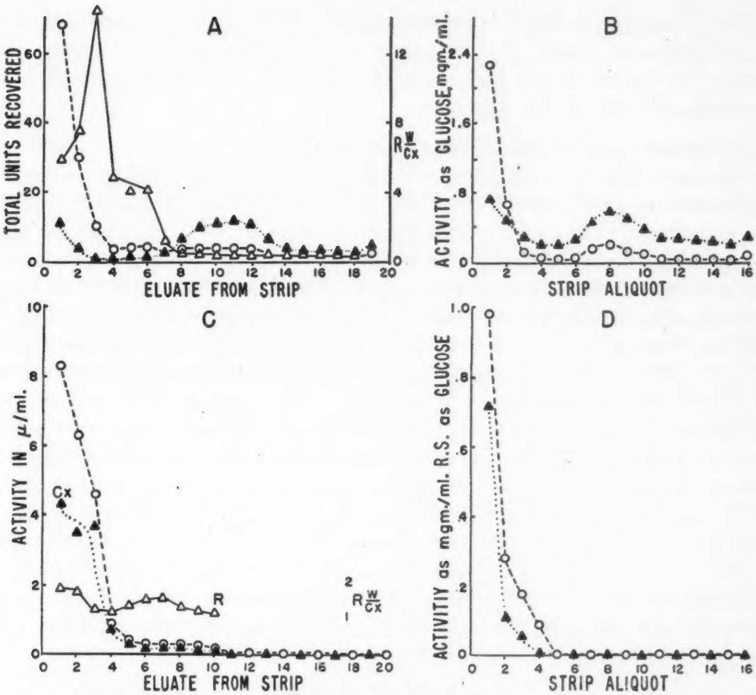

FIG. 2. Paper chromatograms: Comparison of direct assay of aliquots of strip with assays of eluates.

Activity: W = activity on Walseth cellulose, ○- - -○; Cx = activity on CMC, ▲·····▲; $R\ W/Cx$ = ratio of activities on a unit basis, △———△.

Myrothecium verrucaria. A partially purified preparation precipitated from ethanol. *A.* Eluted from strips before assay. *B.* Direct assay by immersion of paper strip in substrate buffer mixture.

Trichoderma viride. A concentrate obtained by acetone precipitation of the cell-free filtrate. *C.* Eluted from strips before assay. *D.* Direct assay of paper strips.

The use of eluates to determine $R\ W/Cx$ thus permits the detection of three fractions instead of the two evident in direct assays of the paper strips. (Previous work (18) with paper chromatograms of proteins has shown that a certain amount of a homogeneous protein may remain at the initial starting point while the rest moves down the paper. It is not possible, therefore, for us to state definitely that the first fraction is not made up of a mixture of the other two components immobilized at the zero point.) In *T. viride* filtrates (Fig. 2*C*, *D*), there is little movement of the activity from the initial spot even on long development (68 hr.) and there is comparatively little change in the $R\ W/Cx$ value. We would hesitate to claim the presence of more than a single component in *T. viride* filtrates on such data.

It should be pointed out that the accuracy of determination of the very high R values is poor, since the Cx values in these ratios approach zero. A

further limitation is that the results apply only to the eluates and not to the original filtrate. Since *Cx* recovery is always better than *W* recovery, it is certain that more of the high *R W/Cx* fraction, than of the low *R W/Cx* fractions, remains on the paper.

(2) Chromatograms from Cellulose Columns

Several types of cellulose were used in columns in an attempt to separate fractions having different *R W/Cx* values. A *M. verrucaria* filtrate at pH 5.6 was run through the column (Walseth cellulose, 1 part + celite 545, 10 parts) and 12 aliquots of 25 ml. each were collected. The column was then extruded and cut into sections. Each section was eluted at 40° C. with 20 ml. *M*/20 citrate buffer (pH 5.6) for 16 hr. The solids were removed by centrifuging, and the supernatant dialyzed to remove the reducing sugars formed during elution. The chromatogram (Fig. 3) is similar to the paper strip chromatogram of *M. verrucaria* filtrates (in reverse). There appear to be at least three components: (1) the high *R W/Cx* (value about 5.0) is the very slow moving fraction, only a small part of which appears to have been eluted under the conditions used; (2) the low *R W/Cx* (value about 0.70) present in eluates 9–12; and (3) the medium *R W/Cx* (value about 1.7) moving fastest and present in aliquots 4–7. By our criteria the third component appears to be a minor constituent. If we were to test its action against other substrates, however, it might be found to have greater importance. It is evident throughout this work that a fraction of little activity by one criterion may have high activity by another.

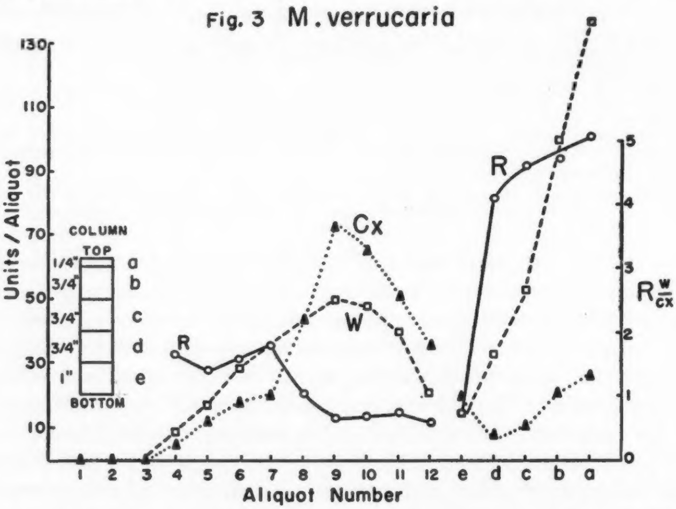

FIG. 3. Separation of cellulolytic components of *Myrothecium verrucaria* on a cellulose column (1 part Walseth cellulose + 10 parts celite).
○——○, *R W/Cx;* □ - - - - □, Walseth activity; ▲ · · · · ▲, *Cx* activity.
M/20 citrate pH 5.6 to wash column and to elute. Recovery *Cx* 55%; *W* 26.4%. There are 12 eluates from the column, plus five eluates of portions of the extruded column.

Alumina (Merck) columns gave some separation of the factors of a *M. verrucaria* filtrate. The first fraction to come through had a low *R W/Cx* value (0.7), later fractions having higher ratios (to 5.0). Filtrates of three other fungi (*T. viride, Penicillium pusillum, Pestalotia palmarum*) showed no separation of components under similar circumstances. Data accumulate which show that the system found capable of separating components of one organism may be worthless for separating those of another.

(3) Chromatograms from Calcium Phosphate Gel Columns

Of the methods tested, the calcium phosphate gel columns have given the best separations. A very low level of salt concentration in the enzyme solution is essential when using this material. With dialyzed preparations, even filtrates of *T. viride* show a multiple component pattern (Fig. 4). A

Fig. 4 T. viride

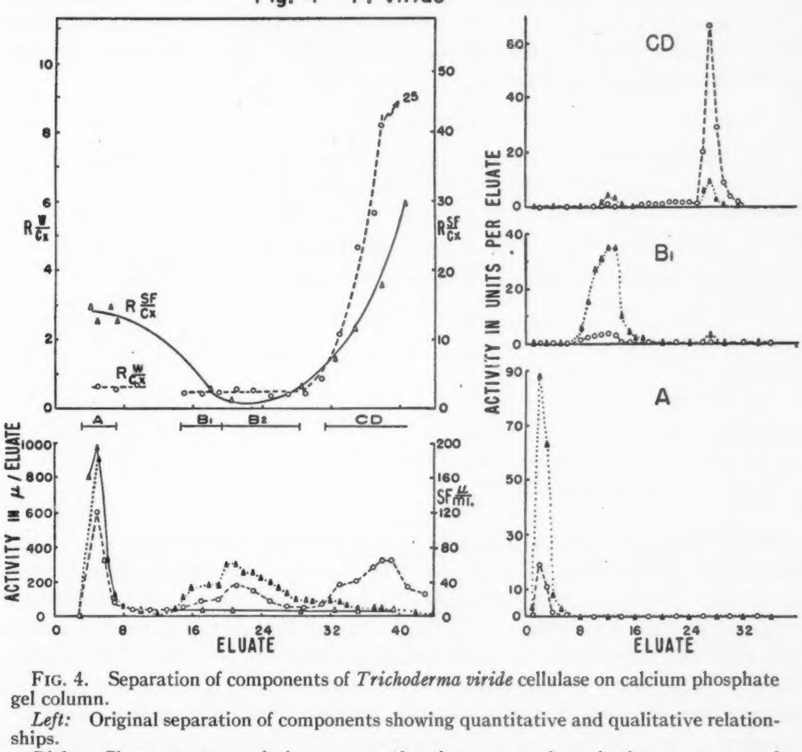

FIG. 4. Separation of components of *Trichoderma viride* cellulase on calcium phosphate gel column.

Left: Original separation of components showing quantitative and qualitative relationships.

Right: Chromatograms of the separate fractions rerun through the same type of column.

Activities: ○ - - - - ○ *W*, on Walseth cellulose; ▲ · · · · ▲ *Cx*, on CMC; △——△ *SF*, on cotton fiber (swelling factor).

Eluting Solutions: 1-3 2% NaCl; 4-15 2% NaCl + 0.002 *M* PO_4 pH 7.0; 16-29 2% NaCl + 0.005 *M* PO_4; 30-43 2% NaCl + 0.02 *M* PO_4.

batch (200 mgm.) of crude enzyme was put through a large column, and the aliquots collected and assayed. Several aliquots representing a particular fraction were combined, pervaporated,* and dialyzed. When rechromatographed, each fraction behaved essentially as it did in the original mixture, though trace amounts of *B* are present in *CD*. The third peak is labelled *CD* because the rapidly changing *R W/Cx* and *R SF/Cx* values over the portion of the curve containing these components would indicate that it is a mixture.

The movement of protein coincides with the movement of enzyme, the peaks (spectrophotometrically at 278 mμ) being associated with the components *A*, and *CD*. Based on Folin protein determinations, the Walseth activity per mgm. protein is of the same magnitude for the three fractions (Table II). *Cx* μ/mgm. values of fractions *A* and *B* are nearly equal, but drop to a very low level in fraction *CD*. (In two similar experiments, however, the *B* fractions had *Cx* μ/mgm. and *W* μ/mgm. values over twice those of the *A* fractions.) The values of *SF* μ/mgm. protein become progressively lower from fraction *A* to fraction *B* to fraction *CD*. The differences between fractions are summarized below.

TABLE II

PROPERTIES OF THE COMPONENTS OF A *T. viride* FILTRATE (OBTAINED BY SEPARATION ON A CALCIUM PHOSPHATE COLUMN)

Fraction	Units per mgm. Folin protein			*R* values	
	Cx	*W*	*SF*	*W/Cx*	*SF/Cx*
A	88	41	1115	0.47	12.7
B	94	32	103	0.34	1.1
CD	3	31	32	10.3	10.7
Original solution	51	79	909	1.55	17.8

(*Protein values are approximate.*)

While the data indicate separation of cellulolytic components of *T. viride* from each other, it must not be assumed that the fractions are free of other materials. β-glucosidase (cellobiase) is present in the extracellular growth medium in trace amounts. Concentration and fractionation on the column result in the appearance of these traces in fraction *A* only. The amount, however, is so low (compared to other types of activity) as to be of no importance to the interpretation of our data. A trace of amylase is also present in fraction *A*, but its movement does not coincide with that of the cellulolytic component (i.e. *Cx* peak in aliquot 4, amylase peak in aliquot 5, both of fraction *A*).

* *Pervaporation is a means of concentrating a solution within a dialysis membrane by blowing air over it.*

Filtrates of a number of organisms have been passed through the calcium phosphate gel columns. Because *M. verrucaria* has been employed in much of the work on cellulolytic enzymes, its pattern is included here (Fig. 5). The patterns for filtrates of *Stachybotrys atra*, and of *Pestalotia palmarum* resemble that of *M. verrucaria*. The simplest chromatogram obtained on this type of column is that of *Penicillium pusillum* which behaves as a single component.

While activities of the various types are usually at maximum (or minimum) values in the same eluates, this is not always so. In the *M. verrucaria* chromatogram, there is one *Cx* peak in eluate 25, but the corresponding Walseth peak is in eluate 27. A similar observation was made on the *Pestalotia palmarum* filtrate.

Fig. 5 M. verrucaria

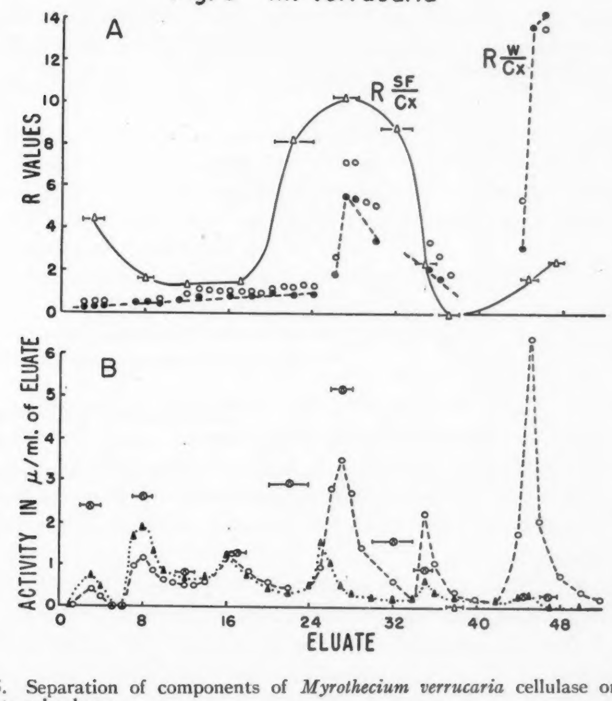

FIG. 5. Separation of components of *Myrothecium verrucaria* cellulase on calcium phosphate gel column.

A. Nature of fractions as determined by their *R SF/Cx* and *R W/Cx* values: *R W/Cx* ● – – – – ●, values based on dialyzed eluates; *R W/Cx* ○ ○, values based on undialyzed eluates; *R SF/Cx* △——△, values based on dialyzed eluates.

B. Distribution of activities in the various eluates: ○ – – – – ○ *W*, on Walseth cellulose; ▲ · · · · ▲ *Cx*, on CMC; ⊢Ⓐ⊣ *SF*, on cotton fiber (swelling factor), the lines indicate the eluates combined for each determination.

Eluting solutions: 1-4 2% NaCl; 5-12 2% NaCl + 0.002 *M* PO_4; 13-22 2% NaCl + 0.005 *M* PO_4; 23-32 2% NaCl + 0.01 *M* PO_4; 33-42 2% NaCl + 0.02 *M* PO_4; 43-52 2% NaCl + 0.05 *M* PO_4; pH 7.0.

(*C*) *Characteristics of Trichoderma viride Fractions Obtained from Calcium Phosphate Gel Columns*

In addition to differences in relative activities (*R* values), and to characteristics of movement on columns, we have found other interesting differences between the three *T. viride* fractions. All of these findings are based on a re-evaluation of our previous work in terms of the multiple enzyme theory.

(*1*) *Fluidity vs. Reducing Groups*

Since a rapid change in fluidity per unit increase in reducing value might indicate a random type splitting, CMC was hydrolyzed by the various fractions and the data plotted as reported earlier (7). The slopes (ϕ/R.V.) differ for the three fractions (Fig. 6). Since these results could be due to increasing amounts of cellobiase, the three fractions were analyzed for this enzyme. Cellobiase was found in fraction *A* only, and here in such low concentrations as to be ineffective under the conditions of test. Actually if the differences in slope had been due to cellobiase, it is in fractions *B* and *C* and not in fraction *A* that we would have expected to find it.

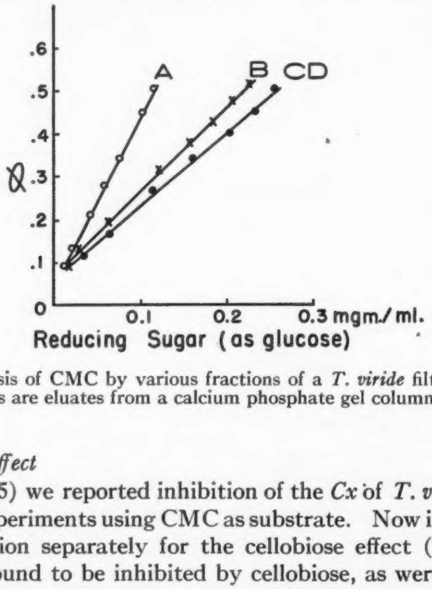

FIG. 6. Hydrolysis of CMC by various fractions of a *T. viride* filtrate (viscosimetric method). Fractions are eluates from a calcium phosphate gel column. Fluidity (ϕ) vs. reducing groups.

(*2*) *Cellobiose Effect*

Previously (15) we reported inhibition of the *Cx* of *T. viride* by cellobiose in viscosimetric experiments using CMC as substrate. Now it seemed desirable to test each fraction separately for the cellobiose effect (Table III). The bulk filtrate was found to be inhibited by cellobiose, as were also fractions *B* and *CD*. On fraction *A*, however, cellobiose had little, if any, effect.

A different filtrate from *T. viride* was stimulated (39%) by cellobiose. Fraction *A* of this filtrate from the phosphate gel column was stimulated to an even greater degree (136%). Fractions *B* and *CD* were inhibited as in the previous test. It appears that pure fraction *A* may be stimulated by cellobiose, and that whether a particular crude filtrate of *T. viride* will be stimulated or inhibited will depend upon the proportional amounts of the components *A*, *B*, and *CD*.

TABLE III

INHIBITION OF VARIOUS *T. viride* FRACTIONS FROM CALCIUM PHOSPHATE GEL COLUMN BY CELLOBIOSE AND BY METHOCEL

Fraction	Cellobiose inhibition of CMC hydrolysis (visc.) in %			Cellobiose inhibition of Wals. Cell. hydrolysis in %		Methocel inhibition of CMC hydrolysis (visc.) in %	
	Cellobiose conc.			Cellobiose conc.		Methocel conc.	
	0.5%	1.0%	2.0%	0.5%	1.5%	0.006%	0.02%
A	+3.0	+5.0	2.0	(12)	33	(+4.0)	0.0
B	25.0	36.0	42.0	(7)	32	35.0	64.0
CD	27.0	NT	51.0	38	61	33.0	63.0
Original	21.0	32.0	34.0			36.0	41.0

Values in ( ) are probably not significant. + indicates stimulation. NT = not tested. The percentage inhibition in viscosity tests is determined from the change in fluidity per unit time ($d\phi/dt$); the inhibition in hydrolysis of Walseth cellulose is based on weight loss experiments.

The hydrolysis of Walseth cellulose is inhibited, but never stimulated, by cellobiose (Reese and Gilligan (13)). Here, fractions *A* and *B* were found to be less inhibited than fraction *CD* (Table III). Fraction *B* behaved like fraction *CD* when CMC was being hydrolyzed, but like fraction *A* when Walseth cellulose was the substrate.

(Recent experiments on different adsorbents show that the cellobiose-stimulated *Cx* does not always accompany fraction *A*. The data given, originally thought to support the difference between *A*, *B*, and *CD*, now seem to point to still another component.)

(*3*) *Methocel Effect*

Methocel had been found earlier to inhibit the *Cx* of *T. viride.* Results of viscosimetric tests (Table III) show that fraction *A* is relatively unaffected by methocel in concentrations exerting appreciable inhibition on *B* and *CD*. The action closely parallels that of cellobiose used under similar conditions (but at different concentrations).

When Walseth cellulose was the substrate, methocel again had little effect on fraction *A*, as compared to its action on fractions *B* and *CD* (data not reported).

(*4*) *Relative Activities on Hydrolyzed Celluloses of Different DP*

Cellulose resuspended from 85% phosphoric acid had a degree of polymerization (*DP*) of about 500. When the cotton was prehydrolyzed with dilute hydrochloric acid, then treated with phosphoric acid, the resulting *DP* was about fifty (end group determination, Meyer (11)). Enzymatic hydrolysis of these two solid celluloses differed for the three *T. viride* fractions used. The activity of fraction *B* on the long chains was 1.6 times that on the short chains, while the activity of fraction *CD* on the long chains was 0.66 times

that on the short. Fraction *A* was nearly equally active (1.1) on both chain lengths. The effect of *DP* on rate of hydrolysis is thus a function of the enzyme fraction under investigation.

(5) *Effect of Enzyme Concentration on the Hydrolysis of Cellulose by the Components of Cellulolytic Filtrates*

A comparison of *T. viride* fractions (Fig. 7) shows that as the enzyme concentration increases, the increment in the rate of activity decreases more rapidly for "*A*" than for "*CD*." This is true for both *W* and *SF* activities. The Walseth activity data are in agreement with the finding (above, C–4) that fraction *CD* is more active on short chains than is fraction *A* (or *B*). It appears (from the nature of the *SF* curves) that this difference in the action of the two components applies also to the very long chain lengths found in cotton.

Comparable *M. verrucaria* fractions behave in the same manner.

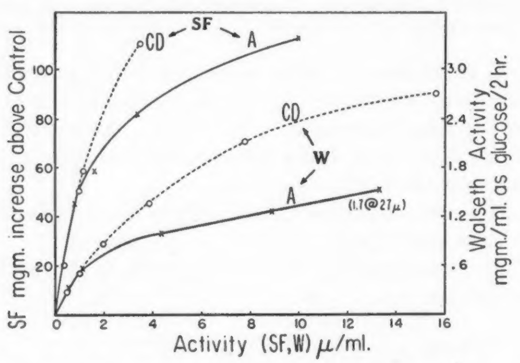

FIG. 7. Effect of enzyme concentration of *T. viride* fractions on Walseth and swelling factor activities. Substrates = Walseth cellulose of low *DP*, and cotton. Temp. = 50° C. Time two hours for Walseth activity; one hour for *SF*.

(6) *Synergistic Effects*

Recovery of the cellulolytic components of a filtrate varies with the type of activity measured. The recoveries from the calcium phosphate gel column are of the following magnitudes: *Cx* 90 + %; *W* 50–65%; *SF* 30%. Since the recovery of protein (Folin) was found to approach 100%, it did not seem likely that activity (*W* and *SF*) was being retained on the column. Either the enzymes were being inactivated, or a synergistic effect present in the whole filtrate was being lost when the components were separated.

The fractions to be compared were diluted to the same unitage basis. Mixtures of fractions thus had the same calculated unitage as each fraction alone. Values above the calculated were considered to show that the fractions were synergistic, and the percentage increase determined from enzyme dilution curves.

A strong synergistic effect is apparent between the first and last fractions, when the activity was measured as swelling factor, or as Walseth activity, but no such effect was found on *Cx* (Table IV). Filtrates of both fungi behaved in the same manner, but only the maximum effect is shown for *M. verrucaria*. As with *T. viride*, the increases obtained with other combinations of fractions were much less. Thus, the apparently poor recoveries of *SF* and *W* activities from columns are not real, but are low because of the *absence* of the synergistic effect in the individual fractions.

TABLE IV

SYNERGISTIC EFFECTS OBTAINED BY COMBINING CELLULOLYTIC FRACTIONS FROM CALCIUM PHOSPHATE GEL COLUMNS

Filtrates	Fractions	% Stimulation = $\left[\frac{\text{(Exptl.-Calc.}}{\text{Calc.}} \times 100\right]$		
		Walseth activity	*SF* activity	*Cx* activity
Trichoderma viride	*A* + *B*	20.0	37.0	NT
	A + *CD*	110.0	100.0	± 6 (R.S.)*
	B + *CD*	58.0	69.0	NT
Myrothecium verrucaria	First + Last	93.0	127.0	0–6 (Visc.)

* *R.S. = reducing sugar, as glucose.*

When the complementary fractions of *M. verrucaria* were used alternately, rather than together (with inactivation by autoclaving between steps), fraction *A* acted on cellulose residues from *A* and from *CD* at about the same rate. On the other hand, fraction *CD* acted on the cellulose residue from *A* at about 165% of the rate of its action on the residue from *CD*. The synergistic action would thus appear to be a contribution of *A* to *CD* and not vice versa. Similar results were obtained with enzyme fractions from *T. viride*.

Of the several possibilities that the observed synergistic effect might be due to an extraneous factor, the following were tested in the manner stated:

(*a*) *Nonenzymatic components.*—Fractions inactivated by autoclaving had no synergistic effect.

(*b*) *Protein.*—Since the protein effect has been emphasized by Whitaker (20) and since protein may be changed by autoclaving (in the above experiment), bovine plasma albumen was added to the substrate in a concentration found by Whitaker to be stimulatory. There was no stimulation by albumen in our tests, nor was there any change in the synergistic effect when plasma albumen was incorporated in the reaction medium.

(*c*) *Noncellulolytic enzymes.*—Both amylase and cellobiase have been detected in low amounts, but only in fraction *A*. Since stimulation also occurs between fractions *B* and *CD* (where amylase and cellobiase are absent),

it is unlikely that either enzyme is playing a role in the synergistic effect. Moreover, addition of extra β-glucosidase (from *A. luchuensis*) to combinations of fractions did not alter the synergistic effect.

Discussion

Number of Components

Here, the components in the cellulolytic system mean all of the enzymes acting on cellulose to achieve its dissolution to glucose. Usually a β-glucosidase (cellobiase) can be detected. This enzyme appears to be intracellular, though traces of it are found in cellulolytic filtrates. In *A. oryzae*, Jermyn (6) has reported finding a β-glucosidase having the following order of activities: cellobiose > salicin > CMC. This claim that cellobiase may be capable of attacking the long chains found in CMC requires further proof. Jermyn's preparations were mixtures (as he readily admits) and may contain a separate *Cx* in the mixture with his β-glucosidases. While it is known that β-glucosidase activity will extend from the dimer to chains of 6- or perhaps even 10-glucose units (Grassmann *et al.* (5)), it is doubtful that its activity will extend to chains of the *DP* found in CMC (average *DP ca.* 100+).

The "cellulases" on which we have been concentrating are those extracellular enzymes which begin their action on native cellulose and carry the process to that state (or beyond) where the substrate is available to the β-glucosidases. Handicapped by lack of well characterized substrates, we have had to choose methods that appear to cover the range of chain lengths and that can be adapted to short time enzyme assay. The swelling factor (*SF*) is applicable to native cotton and of much importance in a consideration of the first step. It has an advantage in that its action does not depend upon an evaluation of end products, i.e. substances whose production may depend upon the action of additional enzymes. As a result, it can be measured in the absence of other cellulolytic enzymes. Its disadvantage is that we have no idea how it works, or even whether its action is on a β 1, 4 linkage. Walseth activity, measured against Walseth cellulose, is perhaps the best of the three measurements, in as much as the substrate represents solid cellulose of low *DP* and high reactivity. One difficulty is the impossibility of obtaining identical batches of substrate. CMC, while soluble and highly active, has the disadvantage that the substituted groups may interfere to varying degrees with *Cx*'s of different types. In spite of these limitations, the results show that it is possible to use the activities on these substrates to characterize various components of cellulolytic mixtures. That they are adequate to detect all of the components seems unlikely.

The evidence for multiple components in the cellulase complex rests on the following differences observed between fractions:

(1) The rates of movement on paper strips, and on various types of columns. (2) The relative activities on the three test substrates (i.e. *R* values). (3) The synergistic effects. (4) The effects of cellobiose and of methocel on the rate

of hydrolysis of CMC. (5) The fluidity change per unit change in reducing value during hydrolysis of CMC. (6) The relative activities on cellulose of differing *DP*.

Regarding the number of components in a particular filtrate, we hesitate to make a guess. Improvement in separation techniques and in well characterized substrates will undoubtedly lead to characterization of additional components. Because of the currently available data, we must assume a minimum of two factors (exclusive of cellobiase). These would be represented by the first and last fractions from the calcium phosphate column (for both *T. viride* and *M. verrucaria*). The other fractions have properties falling between those of the extremes. The question is whether these are mixtures of the first and last components only, or whether additional factors are present. We have no doubt that mixtures are the rule rather than the exception. In spite of this, it seems impossible to limit the number of components to two. In *T. viride*, particularly, a third (the "*B*") component appears to be well characterized. While further speculation is not warranted on the basis of current data, we believe that the number of distinct enzymes active in cellulose hydrolysis will be found to be at least as great as that involved in starch hydrolysis.

Why is it that we find several components in a system (*M. verrucaria*) where Whitaker (20) found but one, and found it by a method (calcium phosphate gel column) which we learned in his laboratory ? Several explanations are possible. First, the conditions under which an organism is grown determine the relative amounts of the various components found in the medium (Fig. 1). Our conditions lead to a mixture of components in a solution of relatively high *R* values. Perhaps Whitaker's growth conditions lead to a relative enrichment in one component. Second, diverse methods for measuring activity are necessary to detect the different components. Whitaker used two substrates (solid celluloses) that may have been too much alike to detect the differences that we have observed. This, however, would not account for the fact that he also found that enrichment in cellobiase activity paralleled enrichment in cellulolytic activity. Third, enzymatic activity is a more sensitive and reliable determinant of homogeneity than are physicochemical methods. Whitaker's electrophoretic and ultracentrifugal data indicated a single component. As with column separation, this finding is open to the objection that with closely related proteins, a separation is difficult except under optimal conditions. Recrystallized ribonuclease which was electrophoretically homogeneous was found to consist of two components when subjected to chromatographic fractionation (Martin and Porter (10)). *Inability* to achieve a separation of similar factors can never be final proof of homogeneity. The finding of one condition resulting in separation is sufficient to overcome the negative data of numerous tests.

Action of Components

While the motility and activity values can lead us to assume as many as five enzymes (Fig. 5*A*) in the cellulase complex (exclusive of cellobiase),

further characterization has been possible only with two of these (the first and the last from the calcium phosphate gel column). It may be that while we have extremes in the end fractions, the intermediate components are affected differently from the first and last components by materials such as cellobiose and methocel. Additional criteria are required for a more definite characterization of these.

The "*A*" component (of *T. viride* and of *M. verrucaria*) moves rapidly through the column and has a low *R W/Cx* value. It contains small amounts of β-glucosidase, and of amylase, but the rates of movement of these differ slightly from that of the cellulase. That the β-glucosidase activity is independent of the cellulase is further shown by its enrichment relative to *Cx* when a mixture of the two is passed through a cellulose column (which selectively removes the *Cx*). The "*A*" component of different filtrates of *T. viride* is *stimulated* (or unaffected) by cellobiose during hydrolysis of CMC. Methocel has no inhibitory effect. During the hydrolysis of CMC by the "*A*" component the fluidity increases rapidly per unit increase in reducing value. These properties are consistent with the view that "*A*" acts in a random fashion.

The "*CD*" component of *T. viride* moves most slowly through the column, has a high *R W/Cx*, and is free of β-glucosidase and of amylase. Cellobiose (but no glucose) is found in the hydrolyzates of Walseth cellulose by this component. "*CD*" is strongly inhibited by cellobiose and by methocel. During the hydrolysis of CMC, the fluidity change per unit increase in reducing value is low. The rate of activity on short cellulose chains is greater than on longer ones. "*CD*" resembles β-amylase in the properties described, and may act from the chain ends.

The "*B*" component of *T. viride* resembles the "*A*" component in some properties, the "*CD*" component in others. In only one observed instance does it show an extreme value—i.e. it is relatively most active (of the three components) on chains of high *DP*. It differs also from the other two components in that both the *R W/Cx* and *R SF/Cx* values are low. Further characterization cannot now be made.

The synergistic action (*W*, *SF*) of one component on another, greatest between components "*A*" and "*CD*" (*T. viride*), is a highly interesting finding. That the effect appears to be a contribution of "*A*" to "*CD*" agrees with the belief that the action of "*A*" is random, of "*CD*" endwise. "*A*" just makes more ends available for "*CD*." The absence of a synergistic effect when CMC is the substrate would indicate that a substituted cellulose like CMC is more susceptible to random than to endwise degradation. The high *R* values of "*CD*" support this view (i.e. the ratios are high because the *Cx* activity is low). Synergistic effects between enzymes have rarely been reported. Recently (Williams (21)) a "synergic" action was demonstrated between salivary α amylase and *R* enzyme acting on amylopectin.

Acknowledgments

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CLOSTRIDIUM BOTULINUM TYPE E TOXIN AND TOXOID[1]

By A. L. Barron[2] and G. B. Reed[3]

Abstract

A strain of *Clostridium botulinum* Type E in a beef heart infusion – peptone broth yields moderate amounts of toxin when grown at 30° C. but fails to produce toxin at 37°. When grown in a cellophane sac suspended in a similar medium, at 30° C., there is a 5- to 10-fold increase in the yield of toxin. Toxoid has been prepared from the high potency cellophane produced toxin by clarification with charcoal, Mandler filtration, and detoxification with 0.3% formalin at 30° C. for 20 days. Three doses of the fluid toxoid protects mice against 500 M.L.D. of homologous toxin. One dose of the same toxoid adsorbed on alum affords the same level of protection as three doses of the fluid toxoid. Mixing Type E toxoid with equal amounts of Types A and B *in vitro* augments the antigenic action of the Type E toxoid.

Introduction

A recent survey by Dolman and Chang (2) reports some 20 isolations of *Clostridium botulinum* Type E since this type was first described in 1936 by Gunnison, Cummings, and Meyer (6). In addition to those listed by Dolman and Chang (2) two other isolations were reported in 1937 (10, 11). During the past six years three outbreaks of human Type E botulism have been reported in British Columbia (1, 3, 4) and one in Alaska (12, 1). The Type E organism is probably widespread throughout the world. The organism shows some predilection for fish or other marine products (1, 7, 14).

The severe toxemia and large proportion of fatalities in outbreaks of human botulism caused by Type E suggests that the toxin produced by this type is of a high order of potency. However, all reports of laboratory cultures of Type E organisms indicate very low yields of toxin as compared with cultures of Types A, B, C, and D organisms. The highest toxin titer reported by Dolman *et al.* (3) was 4000 mouse M.L.D. per ml. of crude culture. This is in line with other reports. In contrast Stevenson *et al.* (18) regularly obtained at least 1,000,000 mouse M.L.D. per ml. of crude cultures of Type A. As suggested by Dolman and Kerr (4), man may be more susceptible to this toxin than the test animals which have been used with laboratory cultures. On the other hand the laboratory conditions may not have been conducive to high toxin production.

An attempt has been made to increase the yield of Type E toxin in order to provide material of sufficient potency to permit the preparation of an efficient toxoid.

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Contribution from the Department of Bacteriology, Queen's University, Kingston, Ontario, and the Defence Research Board Kingston Laboratory. Portion of a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree.

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1. Toxin Production

Strains of Type E

In a preliminary comparison of six strains of Type E the VH strain kindly sent to us by Dr. Dolman (Dolman *et al.* (3)) gave consistent yields of toxin. This strain was isolated from pickled herring responsible for an outbreak of human botulism.

Stock cultures were maintained in Robertson's meat as suggested by Stevenson *et al.* (18) for Types A and B.

Media

Several types of fluid media have been tested for growth and toxin production, Table I, at 30° and 37° C. The most satisfactory of those tested both from the point of view of growth and toxin formation was Difco heart infusion supplemented with additional peptone, 1-2%, and glucose, 0.5%. Several different peptones appeared equally effective, Table I. In these media growth is about the same at 30° and 37° C. The pepticase – corn-steep – glucose medium used in this laboratory for the production of Types A and B toxin (Stevenson *et al.* (18)) failed to support active growth of Type E.

TABLE I

GROWTH OF *C. botulinum* TYPE E, VH STRAIN, 24 HR. AT 30° AND 37° C. ON VARIOUS MEDIA

Medium	30° C.		37° C.	
	Growth, 24 hr.	pH 5 days	Growth, 24 hr.	pH, 5 days
Robertson's meat	+++	6.8	+++	6.8
Pepticase – corn-steep – glucose	Nil (48 hr.)		Nil (48 hr.)	
Heart infusion – tryptose – Difco	Nil (48 hr.)		Nil (48 hr.)	
Heart infusion – tryptose – glucose with:				
Difco peptone	+++		+++	5.7
Proteose peptone No. 2	+++		+++	5.3
" " No. 3	+++		+++	5.3
" " No. 4	+++		+++	5.3
Tryptone	+++		+++	5.3
Casitone	+++		+++	5.4
Neopeptone	+++		+++	5.4
Tryptone + 0.5% $CaCO_3$	+++	6.0	+++	6.0

Temperature and Toxin Production

Dolman *et al.* (3) reported yields of toxin by the VH strain up to 4000 lethal mouse doses per ml. of crude culture incubated at 37°. We have been unable to duplicate these yields at 37° in any media tested but good yields have been obtained in cultures incubated at 30° C. In Robertson's chopped meat medium, for example, at 30° the order of 4000 lethal mouse doses of toxin were produced whereas in similar media incubated at 37° though the growth was similar no detectable toxin was formed. This is in agreement with results reported by Gunnison *et al.* (6) and Hazen (8).

Cultivation in Cellophane

The yields of toxin by several species of bacteria have been greatly increased by cultivation in cellophane sacs (Sterne and Wentzel (17), Fredette and Ginet (5), Koch and Kaplan (9)). It has been found in this laboratory that *C. botulinum* Type A grown in pepticase – corn-steep – glucose medium regularly produces the order of a million lethal mouse doses of toxin per ml. of crude culture but when the same strain is grown in a cellophane sac suspended in a similar medium, 40 to 50 million lethal doses of toxin per ml. are produced. Moreover, since the medium used by the growing organism must diffuse through the cellophane, large molecule constituents of the medium are excluded. The crude toxin is therefore not only more concentrated but in purer form.

The usual procedure has been to form a cellophane bag by tying off one end of a length of cellophane tubing which is filled with water or saline and suspended in the medium. After sterilization the content of the bag is inoculated. Sterne and Wentzel (17) improved the procedure by intussuscepting a length of cellophane tubing which somewhat increases the surface of cellophane. In this device the authors obtained yields of Type C toxin some 20 times greater and Type D toxin 100 times greater than they obtained in ordinary culture flasks without cellophane.

Type E toxin has been developed in Sterne and Wentzel's modification of the cellophane technique. Fig. 1 indicates an arrangement to contain 500 ml. of fluid inside the cellophane and 6000 ml. outside the cellophane bag. This cellophane bag unit consisted of $2\frac{1}{4}$ in. seamless cellophane tubing well soaked, invaginated, and tied to the large rubber bung support. A similar but larger form consisted of $5\frac{1}{2}$ in. seamless cellophane tubing invaginated and attached to a stainless steel cover to a large pyrex cylinder. The vessel held 25,000 ml. of culture media and the cellophane sac 2500 ml. of fluid.

The most satisfactory yields of toxin were obtained in these cellophane culture vessels with the following medium:

Heart infusion – tryptose broth, Difco	25 gm.
Glucose	10 gm.
Tryptone	10 gm.
Calcium carbonate	5 gm.
Distilled water	1000 ml.

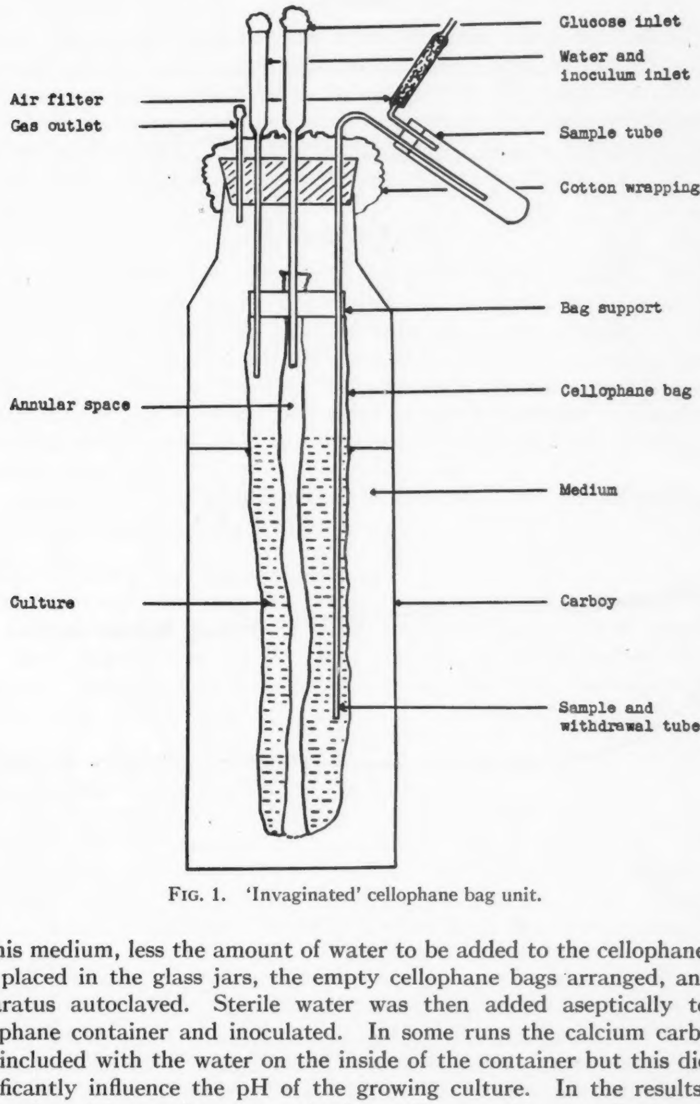

FIG. 1. 'Invaginated' cellophane bag unit.

This medium, less the amount of water to be added to the cellophane bag, was placed in the glass jars, the empty cellophane bags arranged, and the apparatus autoclaved. Sterile water was then added aseptically to the cellophane container and inoculated. In some runs the calcium carbonate was included with the water on the inside of the container but this did not significantly influence the pH of the growing culture. In the results here shown the calcium carbonate was in the medium outside the cellophane.

Cultures in this cellophane apparatus grow rapidly and reach a peak at 36 to 48 hr. In contrast to ordinary cultures, in the cellophane there is little or no autolysis of the cells for a period up to 20 days. The pH of the culture in the bag rapidly becomes acid as indicated in Table II and Fig. 2, while the outside solution, which remains sterile, shows little pH change.

TABLE II

YIELD OF TYPE E BOTULINUM TOXIN IN CELLOPHANE BAG CULTURE

Days' incubation	30° C.	25° to 29° C.	pH
	Toxin, M.L.D./ml.	Toxin, M.L.D./ml.	
½			6.3
1			5.9
3			5.5
5	6×10^3		
6	9×10^3		
7	1.5×10^4	1.5×10^3	5.5
9	1.5×10^4		
10	3×10^4	4×10^3	5.3
15		8×10^3	
18		2×10^4	5.5
20		3×10^4	5.7
24		5×10^4	5.7

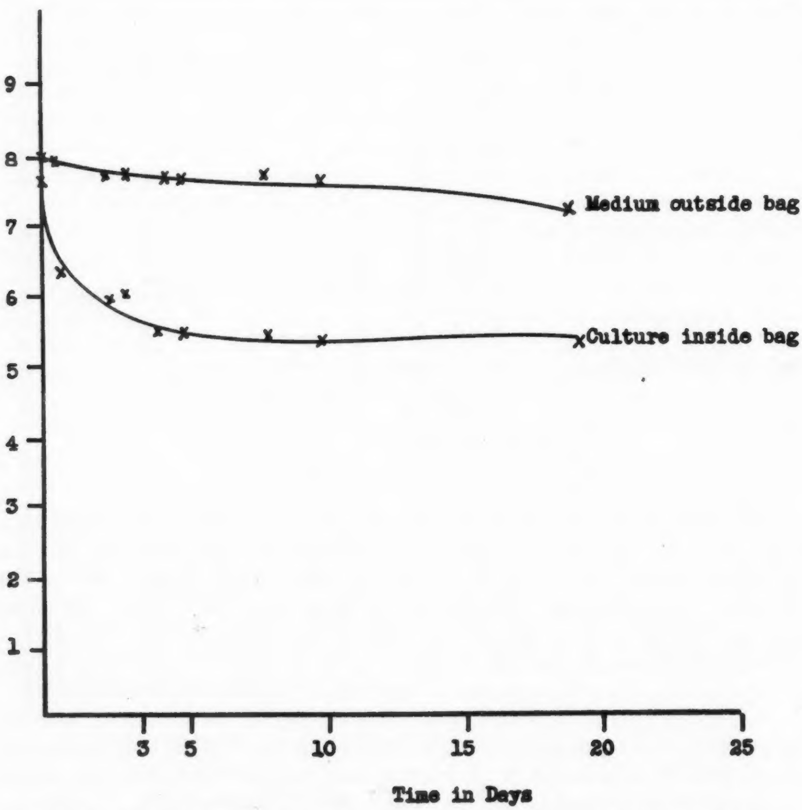

FIG. 2. Changes in pH of culture during growth in 'invaginated' cellophane bag.

Toxin Yields

Toxin assays, Table II, of the crude cellophane bag cultures show an appreciable yield of toxin after five to seven days' incubation which gradually increases to a maximum in 10 to 24 days. Titers ranging from 10,000 to 50,000 M.L.D. for the mouse per ml. were regularly obtained, with an average yield well over 20,000. This yield amounts to at least a fivefold increase in yield over that obtained by conventional culture procedures.

2. Type E Toxoid

Toxoids have been prepared for *C. botulinum* Types A and B (Rice and Reed (16), Nigg *et al.* (13)) and Sterne and Wentzel (17) have produced Type C and D toxoids. Very little has been reported on toxoids for Type E. Gunnison *et al.* (6) mention a Type E toxoid in connection with the classification of strains. Prévot and Huet (14) formalized the toxin from their strain of Type E and used it in the production of anti E serum in rabbits. Aside from these references there does not appear to have been any work done on toxoid for this type.

The preparation of toxoid from the high potency Type E toxin produced in cellophane cultures has been undertaken.

Toxoid from Crude Cultures

The crude cultures from cellophane bags with toxin content of 20,000 M.L.D. per ml. and containing large numbers of intact cells have been formalized and held at 30° C. up to six weeks. Detoxification was not complete, though greatly reduced, in this period. The material showed a very low immunizing value.

Clarification of Cellophane Cultures

Two methods of clarification have been used.

(*a*) Ten-day cultures from cellophane, rich in intact cells and containing 25,000 M.L.D. of toxin per ml. was centrifuged at low speed and the supernatant centrifuged in a Servall vacuum type instrument at 15,000 r.p.m. for 30 min. The final supernatant was clear and free from cells. There was little or no loss in toxicity. As this procedure is not practical for large lots a second method of clarification was undertaken.

(*b*) To cellophane produced toxin, activated charcoal (Darco) was added to a concentration of 2.5%, well mixed, and the mixture filtered through Whatman No. 4 filter paper on a Buchner funnel under slight suction. The filtrate was free of cells, clear, and exhibited little or no loss in toxicity when assayed immediately after clarification. The filtrate was, however, unstable. After several days storage at 4° or 20° C. there was an appreciable loss in toxicity.

Filtration

Toxins clarified by the above methods were sterilized by Mandler filtration. The clarified toxins filtered readily and in sharp contrast to Types A and B toxins there was little or no loss in toxicity. As the charcoal-clarified and

Mandler filtered toxins were unstable, formalization was started immediately after completion of clarification and filtration. It should be noted that in several instances filtrates which had apparently lost a great deal of toxicity during treatment and storage made good toxoids.

Formalization

Formalin, adjusted to pH 6.0, was added to the filtered toxins to a final concentration of 0.3%. This was added slowly with constant gentle stirring by rotation of the bottles. The toxin–formalin mixtures were incubated at 30° C. with gentle rotation of the bottles daily. Detoxification was rapid, after 20 days 1 ml. doses of undiluted toxoid failed to produce any symptoms of botulism in mice.

Alum-precipitated Toxoid

Since the work on Types A and B toxoids indicated a marked improvement in antigenicity when the fluid toxoids were adsorbed on alum (16), both fluid- and alum-precipitated Type E toxoids were prepared. The procedure of alum precipitation was similar to that used by Rice with Types A and B. A 10% sterile solution of aluminium potassium sulphate was added to the fluid toxoid to final concentrations of 0.5% or 1.0%. The precipitate was washed three times with sterile saline and the final washed precipitate suspended in a volume of sterile saline equal to the original volume of fluid toxoid.

Antigenic Response to Fluid and Alum Toxoids

The antigenic value of the toxoids was determined by giving mice a single 1 ml. dose of the toxoid or a series of doses at intervals of 10 days. Twenty-one days after the single dose or after the last dose the mice were challenged with a dose of Type E toxin. Table III summarizes results obtained with the immunization of mice with fluid toxoid. It is apparent that the fluid toxoid prepared by charcoal clarification is as good as the more laborious centrifuge clarified preparation. It is also apparent that one or two doses of the fluid toxoid produces a relatively low immune reaction in mice whereas three doses produce a good level of protection.

TABLE III

IMMUNIZATION RESPONSE OF WHITE MICE TO FLUID TYPE E BOTULINUM TOXOID CHALLENGED WITH HOMOLOGOUS TOXIN

Toxin clarified by	No. doses toxoid	Days after last dose	Challenge M.L.D.	No. mice surviving
Charcoal adsorption	1	21	10	1 of 15
	2	21	100	3 of 11
	3	21	100	7 of 7
	3	21	500	11 of 12
Centrifugation H. S.	2	21	10	7 of 8
	3	21	100	4 of 6

Table IV summarizes results obtained with alum-precipitated toxoids. It is apparent that, as in the case of the fluid toxoids, there is no significant difference between toxoids prepared by high speed centrifuging and by charcoal clarification. Animals given a single dose of alum-precipitated toxoid and challenged 10 days later showed a relatively low level of immunity whereas similarily immunized animals challenged 21 days after immunization had a very much higher level of immunity. It is also evident from the table that the toxoids precipitated with 1% alum are somewhat superior to those prepared by precipitation with 0.5% alum.

TABLE IV

IMMUNIZATION RESPONSE OF WHITE MICE TO TYPE E BOTULINUM ALUM TOXOID CHALLENGED WITH HOMOLOGOUS TOXIN

Toxin clarified by	% alum	No. doses	Days after last dose	Challenge M.L.D.	No. mice surviving
Centrifugation H. S.	0.57	1	10	10	1 of 8
		1	21	10	8 of 8
		1	21	50	3 of 8
	1.0	1	10	10	0 of 10
		1	21	500	10 of 10
		2	21	5000	1 of 10
Charcoal adsorption	0.5	1	21	50	11 of 11
		3	29	10000	1 of 11
	1.0	1	10	1000	19 of 35
		1	21	1000	9 of 10
		1	21	5000	4 of 13
		2	21	10000	1 of 12

A comparison of Tables III and IV indicates that a single dose of alum-precipitated toxoid provides as high or a higher level of immunity than provided by two or three doses of similar toxoid in the fluid state.

Polyvalent Toxoid

Rice (15) found that Type A toxoid provided a high-level specific immunity; the Type B toxoid produced only a low specific immunity. When the A and B toxoids were mixed in equal proportions and injected into mice the immune response to A toxin was similar to that in animals receiving the A toxoid alone but the immunity to B toxin was much greater in the animals receiving the mixed A and B toxoids than in animals receiving B toxoid alone.

Alum-precipitated A, B, and E toxoids were mixed in equal proportions and 1 ml. doses injected into mice. Another group of mice were given E toxoid only from the same lot as used in the A–B–E mixture. Ten and 21 days after the last dose both lots were challenged with Type E toxin. The results are shown in Table V. It is apparent from the table that the animals receiving the polyvalent A–B–E toxoid exhibited a somewhat higher specific Type E immunity than the animals receiving the E toxoid only. The

difference is most conspicuous in the animals challenged 10 days after the immunization. It seems therefore apparent that the A toxoid or the A and B toxoids somewhat improve the immunizing value of the E toxoid. This is in line with Rice's finding that Type A toxoid improves the immunizing value of B toxoid.

TABLE V

COMPARISON OF IMMUNIZATION WITH TYPE E ALUM TOXOID AND MIXTURES OF BOTULINUM TOXOIDS AGAINST TYPE E TOXIN

Toxoid	No. doses toxoid	Days after last dose	Challenge M.L.D.	No. mice surviving
Type E	1	10	1000	8 of 15
	1	21	5000	4 of 13
	2	21	10000	1 of 12
(A – B) – E mixed *in vitro*	1	10	1000	13 of 15
	1	21	5000	4 of 10
	2	21	10000	3 of 15
(A – B) – E mixed *in vivo*	1	10	1000	1 of 15
	1	21	5000	1 of 15

In Vivo Mixture of Toxoids

In a third group of mice, immunized at the same time as the two groups mentioned in the previous paragraph, the animals were given 0.66 ml. of the mixture of A and B toxoids and five hours later were given 0.33 ml. of Type E toxoid. These are the same amounts of the same lots of toxoids as given when the toxoids are mixed before injection. These animals were challenged with E toxin in the same way as those given the mixture. The results are shown in the last section of Table V. It is apparent from the table that this *in vivo* mixing of toxoids results in complete loss of the adjuvant effect of *in vitro* mixing.

Conclusion

It is apparent from the results shown in this paper that the nutritional and environmental conditions supporting growth and toxin production of *C. botulinum* Type E differs widely from Types A and B.

Growth of Type E in cellophane sacs greatly augmented toxin production, as in the case of Types A, B, C, and D. The toxic crude culture has been clarified by centrifuging or by charcoal. Clarified cultures passed a Mandler filter without loss of toxicity. The filtered cultures were readily converted to

toxoid with formalin at 30° C. The toxoid was highly antigenic but adsorption on alum improved the antigenicity. Mice immunized with the toxoid exhibited a high level of specific immunity to Type E toxin.

When Type E toxoid was mixed with Types A and B toxoids, a higher level of immunity against Type E toxin was produced in mice than when the Type E toxoid was administered alone. This adjuvant effect was apparent when the toxoids were combined *in vitro* but not when the toxoids were injected individually at five-hour intervals.

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AN ANTIBIOTIC-PRODUCING BACTERIUM OF THE GENUS *PSEUDOMONAS*[1]

By S. H. F. Chinn[2]

Abstract

A Gram-negative rod, conforming to *Pseudomonas viscosa* (Frankland and Frankland) Migula, was isolated in practically pure culture from a sample of wheat that did not show the usual mixture of epiphytes. *In vitro* studies revealed an unusual antibiotic spectrum against a variety of Gram-positive and -negative bacteria as well as against *Helminthosporium sativum* and *Fusarium culmorum*. Comparative studies of the organism and *P. aeruginosa*, *P. fluorescens*, and *P. chlororaphis* indicated that it possessed greater antibiotic activity than any of these three species of *Pseudomonas*. Application to the control of some plant pathogenic organisms is suggested.

Introduction

In the course of a study of the common root-rot problem of wheat, a Gram-negative rod possessing a high degree of antagonism towards the causal pathogens, *Helminthosporium sativum* P.K. and B. and *Fusarium culmorum* (W.G. Sm.) Sacc., was obtained. The bacterium designated as C50 was isolated from a head of Sevier wheat. Preliminary studies indicate that it possesses a number of interesting and promising characteristics. Under normal conditions the microflora of wheat grain is quite varied; James *et al.* (3), in their study of six replicates of four samples of wheat, found that although certain species of bacteria were predominant, numerous types and species of other bacteria, yeasts, and fungi were also present on the grain. The author in his numerous bacteriological samplings of wheat also found, except in the case where C50 was concerned, the existence of a varied flora on the grain. In this particular instance only two types of bacteria were observed and C50 appeared in a ratio of more than 9 to 1.

The C50 culture was studied for its antibiotic activity. Results showed that it was antagonistic towards nearly all the test organisms used in the preliminary trials. Further experiments were carried out to test the culture for its ability to control covered and loose smut of barley and common root rot of wheat. As will be reported in a later paper, treatment of seeds with a broth culture of C50 was effective in the control of these three plant diseases.

In view of these findings, it was decided that further studies should be made of the culture. The present paper consists of an attempt to classify the organism and a study of its antibiotic potential as measured by its antagonism against a variety of microorganisms. Its antibiotic activity is also compared with those of three known species of *Pseudomonas*, namely: *P. aeruginosa*, *P. fluorescens*, and *P. chlororaphis*.

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Contribution No. 370 from Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa.
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Methods

Cultural Characteristics of Organism C50

The methods used for characterization of the culture were, in general, those described in the Manual of Methods for Pure Culture Study of Bacteria (6).

Antibiotic Activities of C50

In the analysis of the culture for its antibiotic activities, 12 species (17 strains) of bacteria were used. The determination was made with the cross-streak technique (8) using nutrient agar.

Another rather crude test was carried out to determine whether air contaminants can establish themselves in a broth culture of C50. Besides C50, about 100 other cultures of various species of bacteria and actinomycetes, which were isolated during the screening of organisms antagonistic to *H. sativum*, were grown in separate flasks of a yeast extract mineral broth. After an initial incubation of five days at room temperature, the cotton plugs were removed from the flasks and the cultures exposed in the laboratory for periods extending up to three months. The level of liquid in the various flasks was maintained by addition of tap water. Cultures were recorded and discarded when macroscopic growth differing from that of the original cultures was observed.

In the comparative study of the antibiotic activities of C50 and the three antibiotic-producing species of *Pseudomonas*, 11 test organisms composed of seven saprophytic bacterial species, two human pathogenic species, and two phytopathogenic fungal species were used. The antagonistic effects of the growth of the antagonistic cultures against the 11 test organisms were determined by using a modification of the spot-inoculation technique (4). A yeast-extract mineral agar of the following composition was used: glucose, 20 gm.; yeast extracts, 5 gm.; ammonium sulphate, 2 gm.; dipotassium phosphate, 1 gm.; magnesium sulphate, 0.01 gm.; tap water, 800 ml.; soil extract, 200 ml.; and agar, 15 gm. The antagonists, four to a plate, were spot-inoculated on the agar. After three days' incubation at 24° C. each plate was flooded with 3 ml. of an agar medium which was seeded with a test organism. Nutrient agar was used for the seeding of the bacteria and potato dextrose agar for the fungi. After further incubation, 24 hr. at 37° C. for the plates seeded with the human pathogens and four days at 24° C. for the rest of the plates, examination for antagonism was made by measuring the width of the zones of inhibition. It should be noted that the width of the clear zones, rather than the diameter of the zones of inhibition, was used since the measurement of the diameters of the colonies of the antagonists varied to quite an extent.

Results

Morphology

C50 occurred as Gram-negative, non-sporeforming, non-capsulated, and non-acid-fast rods. The ends were rounded and the cells measured 0.5 by 1.0 to 2.0μ. These rods had a tendency to agglutinate on glass slides. The cells were motile and possessed two to four delicate polar flagella which were three times the length of the cells. At times, the flagella appeared to be bipolar.

Growth Characteristics

Colonies on nutrient agar after 72 hr. incubation at 23° C. were circular with a diameter of 3 to 4 mm., entire, smooth, and slightly raised. They were fluorescent with a greenish-yellow pigment diffusing into the agar. In ultraviolet light, young colonies glowed with a bright, bluish-green fluorescence. Older colonies became translucent and lost their fluorescence. Agar slant culture was filiform, smooth, with a pale straw color, becoming effuse and translucent. A moist, chocolate-brown, and somewhat viscid growth was formed on potato plugs. In gelatin, stratiform liquefaction was observed. There was no reduction or coagulation in litmus milk but peptonization occurred after one week. In nutrient broth turbidity with a light surface pellicle was formed. The culture grew between 2° and 36° C. with an optimum of 23° C. It was a facultative aerobic organism.

Biochemical Reactions

No acid or gas was produced from glucose, sucrose, lactose, maltose, or mannitol. A trace of acid was produced from arabinose and definite acidity from xylose. Citrate was utilized and nitrates were not reduced but ammonia was formed. Both the acetylmethylcarbinol and methyl red tests were negative and indol was not formed. Oxidase was produced and the culture was lipolytic. Starch was not hydrolyzed and hydrogen sulphide was produced.

Antibiotic Activities of C50

The antibiotic activities of C50 where the cross-streak technique was employed are summarized in Table I. The culture was active, but in different degrees, against all the test organisms. There was no apparent selectivity although some of the *Bacillus* organisms were inhibited to a greater extent than the others. A relatively high degree of antagonism was attained against *Salmonella typhosa* but the degree of inhibition against *Mycobacterium* was rather low.

In the test where broth cultures of various species of bacteria and actinomycetes were exposed to air contaminants, fungal growth was observed after three to seven days' exposure in all but the C50 and two other flasks out of a total of approximately 100 flasks. No foreign growth was observed in these three flasks during the entire three-month period.

TABLE I

ANTIBIOTIC SPECTRUM OF CULTURE C50
(CROSS-STREAK TECHNIQUE)

Collection number*	Test organism	Zone in mm.†
490	*Micrococcus pyogenes* var. *aureus*	6.0
117	*Escherichia coli*	9.0
499	*Pseudomonas aeruginosa*	8.0
421	*Salmonella typhosa*	14.5
498	*Shigella alkalescens*	9.0
497	*Proteus vulgaris*	11.0
475	*Serratia marcescens*	3.5‡
474	" "	5.5‡
493	*Sarcina lutea*	3.0
818	*Bacillus cereus*	5.0
826	" "	6.5
381	" " var. *mycoides*	24.0
246	" *subtilis*	14.0
825	" *megaterium*	10.0
546	*Mycobacterium tuberculosis* (var. *hominis*) (607)	1.0
749	*Mycobacterium tuberculosis* (607R)	1.0
726	*Mycobacterium tuberculosis*	4.0

* *Collection number, Bacteriology Division, Science Service, Ottawa.*
† *Zone of inhibition expressed as average of duplicate tests.*
‡ *Pigmentation reduced in first 3 mm. of growth.*

The comparative antibiotic activities of C50, *P. aeruginosa*, *P. fluorescens*, and *P. chlororaphis* on the 11 species of microorganisms are summarized in Table II. The data showed that the growth of C50 inhibited all the test organisms. The degree of activity was high; C50 inhibited completely the development of *H. sativum*, both of the human pathogens and three of the saprophytes, and it also gave measurable degrees of inhibition (5 mm. or over) against the remainder of the test organisms. The spectrum and intensity of antagonism of the three known species of *Pseudomonas* were lower than those of C50. It should be noted that not one of the three exhibited antagonism towards *F. culmorum*, and that *P. aeruginosa* and *P. fluorescens* were less active against *H. sativum*, while *P. chlororaphis* had no influence at all on the growth of the latter fungus.

TABLE II

COMPARATIVE ANTIBIOTIC ACTIVITIES OF C50 AND THREE OTHER SPECIES OF *Pseudomonas* (SPOT-INOCULATION TECHNIQUE)

Antagonistic cultures	Test organisms										
	Bacillus subtilis	*Sarcinia flava*	*Micrococcus pyogenes* var. *aureus*	*Aerobacter aerogenes*	*Pseudomonas fluorescens*	*Serratia marcescens*	*Escherichia coli*	*Micrococcus pyogenes* var. *peoria*	*Bacillus anthracis*	*Helminthosporium sativum*	*Fusarium culmorum*
C50	C	C	C	5½	5	6½	7	C	C	C	5½
P. aeruginosa	8½	C	8	s	3	5½	4½	9	13	13	0
P. fluorescens	2	C	7	0	0	2	s	3	6	7	0
P. chlororaphis	3	C	4½	1	2½	s	0	2	7	0	0

Note:—*Results of above measurement are in mm.; they are averages of duplicate tests.*

C—*indicates no growth of test organism in that quarter of the plate influenced by the antagonist.*
s —*indicates slight growth of the test organism in the zones of inhibition; the zones were usually less than 1 to 2 mm. in width.*
0 —*indicates no inhibition of test organism.*

In the above experiment a few plates were kept for about a week after the readings were taken. At that time prolific formation of greenish crystals was observed in the area adjacent to C50.

Discussion

In the morphological and physiological tests C50 agreed in general with the description of *Pseudomonas viscosa* (Frankland and Frankland) Migula as given in Bergey's Manual (1). A slight difference in optimum growth temperature as well as other minor differences in the description of growth characteristics may be noted. *P. viscosa* was reported as motile with presumably polar flagella. Slight variations in description may be expected since materials and techniques used for classification 50 years ago differ to some extent from those of today. In view of the unavailability of cultures of *P. viscosa* comparative studies are not possible. Close agreement with this organism appears to justify considering C50 as specifically identical.

Two properties of the culture are of special interest; the broad and non-selective spectrum of activity as demonstrated by the fact that all of the 28 test organisms in Tables I and II were inhibited, and the marked antagonism shown towards a number of the test organisms. Of special interest and

importance is the antifungal property of the culture as shown by its antagonism towards *H. sativum* and *F. culmorum* and also by the inability of air contaminants to establish themselves in broth cultures of C50.

The latter point recalls the circumstances under which C50 was isolated, which were unique in that the microflora of the sample of grain consisted of only two species of bacteria with C50 appearing as an almost pure culture. It is probable that the organism was a contaminant of the grain rather than an epiphyte since a similar one has not been found in any other bacteriological sampling of wheat. As soon as C50 was established on the grain, its antagonistic properties presumably inhibited the development of the epiphytes and any other subsequent contaminants. If this actually occurred, the application of antagonistic organisms resembling C50 to the control of various plant diseases should not be overlooked.

Results obtained in the comparative studies of the four species of *Pseudomonas* indicate that C50 possesses the greatest antibiotic potential. Its wide spectrum, differing as it does from those of the other pseudomonads, and its more intense antibiotic effect suggest differences in the antibiotics formed though this can only be determined by further investigation.

Numerous studies have been made on the antibiotic potential of various species of the genus *Pseudomonas*. *P. pyocyanea* (*P. aeruginosa*) was mentioned by Florey *et al.* (2) as possessing a wide spectrum and Stokes *et al.* (7) found that the pigments, pyocyanine and hemipyocyanine, produced by the above organism, were inhibitory to a number of dermatophytic fungi. The fact that C50 inhibited *H. sativum* to a greater extent than did *P. aeruginosa* and had some effect on *F. culmorum* points to a difference in the composition of antibiotics produced by the two species of *Pseudomonas*.

According to a study made by Lasseur in 1911 as reported by Florey *et al.* (2), chlororaphin, a greenish pigment formed by *P. chlororaphis*, separated out in culture medium. This fact points to some similarity to the formation of crystals in agar by C50. Whether the difference in the antibiotic spectrum between C50 and *P. chlororaphis* (Table II) indicates that chlororaphin was not the active substance, or that it was formed more rapidly by C50 than *P. chlororaphis* is not known. It is also possible that crystal formation by C50 is incidental to the antagonistic effect or that the crystals formed by the two cultures are not identical.

McIlwain (5) demonstrated that chlororaphin at certain concentrations was inhibitory to *Streptococcus pyogenes;* on the other hand, the author found that C50 had no inhibitory effect on this pathogen. Although the two investigations were not carried out under similar conditions, the difference suggests the probability that chlororaphin and the crystals formed by C50 are not identical.

The interesting spectrum and the intense antagonism exhibited by this organism against the various bacteria and fungi suggest the desirability of further work in the isolation, identification, and evaluation of the active principle produced.

Acknowledgments

The writer is indebted to Mr. G. B. Landerkin for his valuable assistance in the identification and confirmation of certain antibiotic features of the organism, and to Dr. B. J. Sallans and others of the Plant Pathology Laboratory, Saskatoon, and Dr. A. G. Lochhead for their suggestions and critical reading of this manuscript.

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ISOLATION AND PROPERTIES OF RIBONUCLEIC ACID FROM *PSEUDOMONAS HYDROPHILA*[1]

By K. K. Reddi[2] and R. W. Watson

Abstract

A procedure for the isolation of pentose nucleic acid (PNA) from *Pseudomonas hydrophila* has been outlined. The purified PNA is free from protein and desoxypentose nucleic acid (DNA). The pentose component of the PNA has been identified as ribose by conversion to its *p*-bromphenylhydrazone. Adenine, guanine, cytosine, and uracil were found in molar ratios of 1.00, 1.55, 0.91, and 0.55 respectively.

Introduction

Pentose nucleic acids have been characterized from yeast (8), tobacco mosaic virus (4, 16), pancreas (14), and from liver (5). Recently Parsons has presented presumptive evidence, based on chromatography, that the pentose nucleoprotein of *Clostridium welchii* contains ribose (18). The objective of the present investigation has been to characterize the pentose in bacterial PNA from a Gram-negative organism grown on xylose as the sole carbon source.

Experimental

Materials

Pseudomonas hydrophila, N.R.C. 492, originally isolated by Reed and Toner (19) was grown in a synthetic medium with xylose as the sole carbon source (11). The cells were harvested in a Sharples supercentrifuge at 50,000 r.p.m., washed four times with cold 0.85% (w/v) aqueous sodium chloride, then successively with 50%, 80%, 95%, and absolute alcohol, and finally with ether. The ether was removed at room temperature, and the final product was dried *in vacuo* over anhydrous calcium chloride. (Yield 39.8 gm.)

Pure *p*-bromphenylhydrazone was prepared by crystallization of the Eastman free base from water, followed by vacuum sublimation and recrystallization from ether. The pure white compound (m.p. 105°-106° C.) was stable indefinitely in sealed tubes *in vacuo* out of contact with light at 0° C. Desoxyribonuclease was a once-crystallized preparation obtained from Nutritional Biochemicals. All other chemicals were reagent grade.

Isolation and Purification of Pentose Nucleic Acid

The procedure for the isolation and purification of PNA included: (1) extraction with dilute alkali and precipitation with alcohol containing hydrochloric acid, (2) treatment of the crude preparation with desoxyribonuclease,

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(3) denaturation of the proteins with Sevag's reagent, (4) precipitation with alcohol containing hydrochloric acid, (5) dialysis, and (6) further purification by precipitation with glacial acetic acid.

The dried cells were extracted according to the method of Sevag *et al.* (21). A mixture of 10 gm. of the dried cell preparation in 300 ml. of 0.05 *N* sodium hydroxide was incubated at 55° C. for one hour with occasional stirring. The suspension was then cooled to room temperature, centrifuged at 14,000 r.p.m., and the residue again extracted as above. After the pH of the combined supernatants was adjusted to 5.6 with acetic acid, the insoluble material was centrifuged down and discarded. The supernatant, concentrated under reduced pressure at 45° C. to about 50 ml., was filtered, and the clear filtrate added with vigorous stirring to 100 ml. absolute ethanol containing 0.5 ml. concentrated hydrochloric acid. After the precipitate was held for one hour at 4° C., it was centrifuged and washed successively with 70%, 95%, and absolute ethanol and finally with ether. The yield after the final drying *in vacuo* over anhydrous calcium chloride was 0.95–1.30 gm. The procedure for the purification of this crude nucleic acid is outlined in Diagram I.

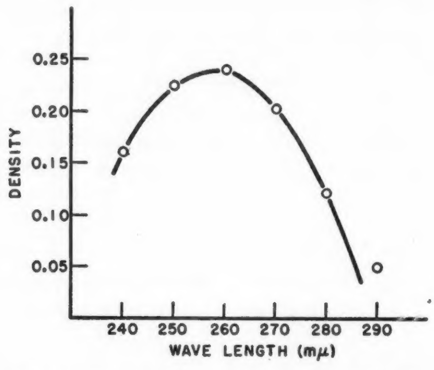

FIG. 1. Ultraviolet absorption spectrum of the ribonucleic acid from *Pseudomonas hydrophila*. (Concentration—0.0024% RNA in phosphate buffer 0.005 *M*, pH 7.2.)

Properties

The pentose nucleic acid prepared according to the procedure in Diagram I was a light white powder, slightly soluble in water giving an acidic solution, and readily soluble in weak alkali. It gave a negative diphenylamine test for desoxyribose (6), a strong positive orcinol reaction for pentoses (17), and negative tests for protein.

The absorption spectrum was measured in a Beckman spectrophotometer according to the method of Davidson and Waymouth (5). Twelve milligrams of the purified preparation was dissolved in a minimum amount of dilute alkali and diluted to 500 ml. with 0.005 *M* phosphate buffer (pH 7.2). The curve (Fig. 1) shows the absorption maximum in the region of 260 mμ.

Diagram I

PURIFICATION OF PENTOSE NUCLEIC ACID

CRUDE NUCLEIC ACID: 2 GM.
TRITURATED WITH 80 ML. SODIUM HYDROXIDE: CENTRIFUGED

Supernatant; 74 mgm. $MgSO_4.7H_2O$ in 5 ml. water, and 10 mgm. desoxyribonuclease in 5 ml. water added, mixture incubated at 25° C. for four hours, a mixture of 18 ml. chloroform in *n*-octanol then added, shaken for one hour and centrifuged; supernatant shaken five times with chloroform:octanol and centrifuged

Residue (discarded)

Supernatant added with vigorous stirring to 140 ml. absolute ethanol containing 0.7 ml. concentrated hydrochloric acid, centrifuged

Residue (discarded)

Supernatant (discarded)

Residue washed with 66% alcohol, suspended in 5 ml. distilled water, dialyzed against running tap water (3° C.) for 10 hr. and against several changes of distilled water at 0° C. for 20 hr., centrifuged

Supernatant (discarded)

Residue washed first with alcohol then with ether and dried; yield 820 mgm. of which 800 mgm. was dissolved in 20 ml. distilled water with minimum of ice-cold 0.1 *N* NaOH; pH of mixture lowered from 7.2 to 6.5 with acetic acid, centrifuged

Supernatant: poured with vigorous stirring into 10 volumes of glacial acetic acid and centrifuged

Residue (discarded)

Supernatant (discarded)

Residue washed and dried with alcohol and ether. The dry powder (562 mgm.) was dissolved in distilled water with minimum ice-cold 0.1 *N* NaOH and precipitated with glacial acetic acid. After being washed and dried in alcohol and ether the precipitate weighed 389 mgm.

Products of Hydrolysis

The purine and pyrimidine composition was determined according to the method of Wyatt (24). One hundred milligrams of the above preparation was hydrolyzed with 1.6 ml. perchloric acid (72%) for one hour at 100° C. After it was cooled to room temperature, the solution was made up to 3.2 ml. with distilled water and centrifuged to remove insolubles. The supernatant was chromatographed for 18 hr. in 65% (w/v) aqueous isopropanol (2 *N* with respect to hydrochloric acid). The separated bases were eluted from the paper with 0.1 *N* hydrochloric acid for 18 hr. and estimated spectrophotometrically. The molar ratios with respect to adenine were: adenine 1.00, guanine 1.55, cytosine 0.91, and uracil 0.55. The purine/pyrimidine ratio is therefore 1.7. The absence of thymine from the products of hydrolysis is additional evidence for the absence of desoxyribonucleic acid (DNA) from the preparation.

Isolation and Characterization of Pentose

Lack of sufficient purified PNA for isolation of the pentose led to a further purification of the crude nucleic acid according to the method of Sevag *et al.* (21). This preparation gave a positive test for desoxypentose. The DNA was removed by incubation with *N* sodium hydroxide for 20 hr. at 37° C. (20) followed by acidification and precipitation with alcohol (22). One gram was then digested with 10 ml. *N* sodium hydroxide at 37° C. for 20 hr. after which the sodium was removed by treatment with Amberlite IR 120 H^+ until the pH reached 4.0. The resin was removed by filtration and washed several times with water. To the combined filtrates one volume of ethanol was added, and the mixture centrifuged. The supernatant was evaporated to dryness at 30° C. under reduced pressure, and the residue was hydrolyzed with 10 ml. *N* sulphuric acid at 100° C. for one hour. The combined supernatants from the centrifuged hydrolyzate were neutralized with barium hydroxide and the precipitate centrifuged and washed twice with water. Concentrated to 10 ml. and treated with Dowex-1-formate to remove nucleotides, the combined filtrate and washings from the resin were finally evaporated to dryness. A 10 ml. aqueous extract of the dried residue was then treated with Amberlite IR 120 H^+ and the filtrates frozen and dried. Of this dried material 10 mgm. was dissolved in 1 ml. of distilled water and chromatographed on paper. The test material, mixed separately with each of the reference sugars, viz. D-arabinose, D-lyxose, D-xylose, and D-ribose, was developed in two solvent systems: (1) ethyl acetate:acetic acid:water (3:1:3) and (2) ethyl acetate:pyridine:water (2:1:2). In both solvents (13) the unknown and the reference D-ribose occupied the same position, which was different from that of the other reference pentoses. Chromatographic evidence therefore indicated that the sugar in the present preparation was ribose.

The freeze-dried material was further purified by descending chromatography on large sheets of Whatman No. 1 filter paper using ethyl acetate: acetic acid:water as the solvent system. The sugar was extracted from the

papers with warm water and the pentose content of the clear filtrates was found to be 16.8 mgm. (17). The filtrates were freeze-dried and a *p*-bromphenylhydrazone derivative was prepared according to the method of Davidson and Waymouth (5). It had a melting range from 164°–168° C., which remained unchanged on admixture with authentic D-ribose-*p*-bromphenylhydrazone.

Discussion

The organism used in these studies was grown in a medium containing xylose as the only source of carbon. The ribose of the ribonucleic acid must therefore have been formed directly or indirectly from xylose. Evidence as to the possible pathways involved may be obtained by growing the organism in a medium containing xylose-1-C^{14}. Lampen (15) has recently shown that ribose-5-phosphate is formed from xylose by cell-free extracts of *Lactobacillus pentosus*, although the mechanism of the conversion is unknown. If the reaction proceeds through a Walden inversion at carbon 3 of xylose, in a manner similar to that reported by Caputto *et al.* (3) for the galactose-glucose transformation, ribose from the PNA of bacteria grown in xylose-1-C^{14} media would be obtained labelled exclusively in the 1-position. However several other pathways are possible for ribose formation from xylose, e.g. cyclic transformations through heptose phosphates (12) or through a C_2–C_3 condensation (1).

The RNA of *P. hydrophila* is characterized by a low uracil and a high guanine content. The existence of quantitative differences in the purine and pyrimidine composition of RNA prepared from different sources has been reported (7). Even in the bacterial cell there may exist qualitatively different types of ribonucleic acid. Working with an enzyme from pneumococci, Thompson and Dubos (23) first presented evidence that ribonucleic acid may be associated with the Gram-positive character of bacteria. Henry and Stacey (9, 10) treated cells of *Clostridium welchii* with a 2% bile solution at 60° C. and were able to remove a surface layer containing the magnesium salt of ribonucleic acid. Pentose nucleotides are known to occur in other parts of the bacterial cell, e.g. in the cytoplasm (2). The ribonucleic acid isolated in the present investigation may therefore come from several parts of the cell and represent an average of types each with a qualitatively different composition. It is of interest that the ratios of adenine to uracil, guanine to cytosine, and purines to pyrimidines are approximately equal, as noted by Elson and Chargaff (7) for *Serratia marcescens* and *Escherichia coli*. Although the numerical values of these ratios differ in the three organisms, and equimolar quantities of purines and pyrimidines do not characterize the bacterial pentose nucleic acids reported, the number of 6-amino groups remains equal to the number of 6-keto groups (7).

The melting points reported in the literature for D-ribose-*p*-bromphenylhydrazone vary from 164°–170° C. D-arabinose-*p*-bromphenylhydrazone melts at 154°–155° C. (5) and the same derivative of D-xylose at 128°–129° C.

(11). The fact that the melting range (166°–170° C.) remained unchanged when authentic D-ribose-*p*-bromphenylhydrazone was mixed with the natural pentose from PNA is conclusive evidence that the pentose present in the PNA of *P. hydrophila* is ribose. Since L-ribose is unknown as a natural product, presumably the sugar is present in the D- form.

Acknowledgment

The *p*-bromphenylhydrazone used in these studies was kindly prepared by W. A. Saunders.

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MICROBIAL PENTOSANASES

I. A SURVEY OF MICROORGANISMS FOR THE PRODUCTION OF ENZYMES THAT ATTACK THE PENTOSANS OF WHEAT FLOUR[1]

BY F. J. SIMPSON

Abstract

A water soluble pentosan extracted from wheat flour was purified by precipitation with ethanol and treatment with pancreatin. Such preparations contained 78 to 82% pentosan and 0.5 to 1% protein. The pentosan of "squeegee" starch also was obtained by removing the starch with pancreatin. The product contained 60 to 75% pentosan and 1 to 3% protein. A number of fungi, streptomycetes, and bacteria produced extracellular enzymes that hydrolyzed the water soluble pentosan. Some of these were tested against the pentosan of "squeegee" starch and found to attack it also. The fungi and streptomycetes possessed adaptive pentosanases whereas 40% of the active bacteria had constitutive pentosanases. Xylose and, to a lesser extent, arabinose stimulated production of the adaptive pentosanases of the molds and streptomycetes while xylose, but not arabinose, was effective with the bacilli. The more active genera were *Alternaria*, *Aspergillus*, *Fusarium*, *Trichothecium*, *Trichoderma*, and *Bacillus*.

Introduction

With the present processes for recovering starch from wheat, difficulty is experienced in obtaining high yields of prime quality starch from crude slurries. About 30 to 40% is lost in a slimy sludge known as "squeegee" starch that is believed to consist of strongly hydrated pentosan masses in which starch granules are embedded (1, 7). Selective enzymatic hydrolysis of the pentosans should release the starch granules and eliminate the "squeegee" fraction. Enzymes of this type have been found in snails (23), in barley, malt, and green malt (5, 18, 23), in wheat (15) and have been obtained from microorganisms (4, 6, 21, 23). This paper describes the results obtained from a survey of the ability of fungi and bacteria to hydrolyze the water soluble pentosan of wheat flour (3, 8, 9) and the relatively water insoluble pentosan of "squeegee" starch. Reports of studies on factors affecting production of these enzymes and their properties will follow in subsequent publications.

Materials

The pentosans were extracted from local commercially milled flours that had not been bleached and to which no improvers had been added. The flours contained about 63–67% starch, 13–14% protein, 1.5–3% pentosan, and 10–12% moisture. In the laboratory, water soluble pentosans were first extracted by the methods of Pence *et al.* (15) and Perlin (16). Some factors affecting extraction and purification of pentosans were studied and are reported briefly below. The fractions used in this survey are described.

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More pentosan could be extracted at pH values higher than 7, but a greater proportion of protein was also extracted. All extractions were therefore done at neutrality or at the natural pH of flour slurries (6.5–6.8). At 60° C., 0.56 gm. of pentosan was extracted from 125 gm. of flour as compared to 0.41 gm. at 30° C.

Aqueous extracts of wheat flour have been reported to contain pentosanases in addition to other enzymes (15). These were inactivated by heating the extracts to 90°–95° C. for three to four minutes (13, 19). The treatment also coagulated 20 to 30% of the protein which was removed along with some pentosan (0.5 to 5%) by filtration.

TABLE I

RECOVERIES OF PENTOSAN AND PROTEIN OBTAINED BY ALCOHOL PRECIPITATION FROM FLOUR EXTRACTS ADJUSTED TO DIFFERENT pH VALUES*

pH of flour extract	Recoveries, % of original		Weight of precipitate, gm.
	Pentosan	Protein	
1	79	0.3	8.2
3	81	2.2	9.8
5	85	3.0	12.2
7	78	2.0	12.9
9	81	1.6	14.3
11	79	0.2	14.2

* *One liter of aqueous flour extract containing 56.4 gm. of protein and 4.8 gm. of pentosan was quickly adjusted at 2–5° C. to the desired pH with concentrated hydrochloric acid or sodium hydroxide and immediately precipitated with 2.4 l. of anhydrous ethanol. The precipitate was rinsed with 70% ethanol followed by acetone and ether and dried in vacuo.*

The optimum pH for precipitating the pentosan with 70% ethanol is about pH 1 (Table I). At this value the greatest reduction in protein occurred and the recovery of pentosan was about equal to that at higher pH values. At pH 11 considerable material was precipitated that was neither pentosan nor protein. This result is reflected in the low pentosan content of the precipitate (Fig. 1) although the amount of pentosan recovered at pH 11 and pH 1 was equal (Table I). Damage to the pentosan molecule by precipitation at relatively high and low acidities was negligible since solutions of the precipitates containing 0.5% pentosan had equal viscosities.

Starch and protein in the precipitated pentosan could be further reduced by the action of enzymes. Pancreatin 3X, trypsin, and pepsin (Nutritional Biochemicals Co.) at concentrations of 0.5% did not affect the viscosity of a 1% solution of crude water soluble

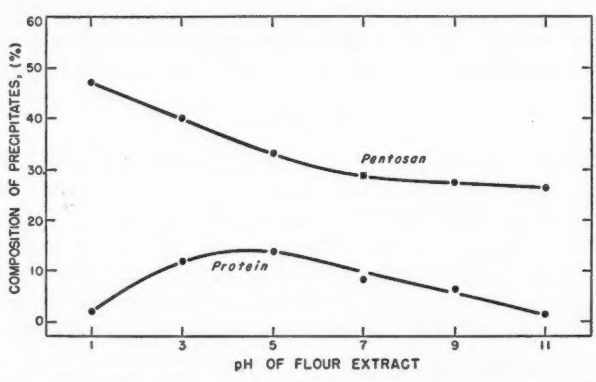

FIG. 1. Composition of the precipitates obtained with 70% ethanol from flour extracts adjusted to various pH values.

pentosan after three hours at 30° C. Papain caused a slight loss in viscosity and a bacterial alpha-amylase preparation (Wallerstein Laboratories) caused a considerable loss. Pancreatin was selected for use since it contained both amylolytic and proteolytic enzymes.

Procedure Used for Extraction and Purification of Water Soluble Pentosans

Water soluble pentosans were obtained from 65 to 100 lb. lots of flour. Although the extractions and concentrations were made with equipment available in a pilot plant the same procedure may be done on a smaller scale with laboratory equipment.

Starch and gluten were separated by the method of Shewfelt and Adams (20) except that the dough was prepared at 60° C. and mixed for 10 min. in a stainless steel tank. Addition of the slurry water lowered the temperature to 30° C. Gluten was removed on a rotex screen and starch in a basket centrifuge. The supernatant, containing water soluble pentosans, was heated to 80° C., cooled to 40° C., and filtered with Celite No. 535 in a closed-delivery plate and frame filter press. A temperature of 80° C. instead of 95° C. was used because of the lower rates of heating and cooling in the larger volumes. The filtrate was concentrated, *in vacuo* at temperatures below 45° C., to 5–7 gal. and stored overnight at 0°–2° C.

The cold flour extract was quickly adjusted to pH 11 with 40% sodium hydroxide and ethanol added immediately to give a final concentration of 70%. The precipitate was washed twice with 70% ethanol, rinsed with acetone, then with ether, and finally air dried. These precipitates contained from 30 to 40% pentosan and 5 to 8% protein.

A 2.5% solution of this precipitate was prepared by stirring the precipitate into hot water containing a minute amount of calcium chloride. The thick solution (pH 7.2–7.4) was heated to 90° C. in a water bath to gelatinize the starch and was cooled to 45° C. To this solution was added 0.2% (w/v) pancreatin that had previously been dissolved in 0.01 *M* sodium chloride and clarified by centrifuging and filtering. When the starch was hydrolyzed (negative iodine test in two to four hours) the pH was raised to 8 to favor proteolytic enzymes. The mixture was stored overnight at 0° C.

A heavy cloudy slime of relatively high nitrogen content usually settled to the bottom of the container. The clearer liquid was carefully decanted, adjusted to pH 1.0–1.2 with concentrated hydrochloric acid, and immediately precipitated with 70% ethanol. A stringy, sticky white precipitate separated. Most of the ethanol, together with some finely dispersed precipitate, was decanted and the remainder removed by filtration. The precipitate was washed with 70% ethanol, acetone, then ether and air-dried. This fraction (*A*) contained 78 to 82% pentosan and 0.5 to 1.2% protein and was used for enzymatic studies.

The finely dispersed precipitate (Fraction *B*) in the decanted ethanol was allowed to settle overnight at 0° C., then filtered, washed, and dried as above. The heavy cloudy slime that remained in the original container was similarly acidified and precipitated. This fraction (*C*) was gray and contained 2 to 6% protein. Fractions *B* and *C* were used in media.

Data from a typical run are given in Table II. About 15 to 25% of the pentosan in wheat flour is usually extracted under these conditions and the remainder may be bound in "squeegee" starch. In the example given, the yield after purification was 10% of that present in the flour. Slightly higher recoveries have been obtained in subsequent isolations.

TABLE II

Yields obtained in a separation of water soluble pentosan from wheat flour

Stage of separation	Amount	Pentosan, gm.	Protein, gm.
Flour	29,500 gm.	650	4120
Concentrated extract	22 l.	90	128
First precipitate	254 gm.	81	19
Total final precipitate	84 gm.	63.9	1.4
Final precipitate:			
Fraction *A*	71	56	0.8
Fraction *B*	4	2.4	0.1
Fraction *C*	9	5.5	0.5

Extraction and Purification of "squeegee" Pentosans

After the gluten has been removed, the initial separation of the "squeegee" fraction from prime starch may be done by settling (7). When the starch was recovered in a large suspended basket centrifuge, the bulk of the "squeegee" fraction lay on top of the hard starch and collected in the upper part of the bowl. The "squeegee" material was removed, diluted with

water, and passed several times at a fast rate through an International desk model basket centrifuge travelling at full speed. Larger basket centrifuges were less effective. When the solid material collecting in the bowl changed from the white color and hard consistency of starch to a gray color and soft consistency, the process was stopped.

The remaining starch was removed by the action of pancreatin. About 0.01% (w/v) pancreatin previously dissolved and clarified and a few crystals of calcium chloride were added to the "squeegee" fraction and the mixture (pH 7.2–7.5) heated to gelatinize the starch. An additional amount of pancreatin (0.2%) was added and the mixture dialyzed at room temperature against 10^{-4} *M* calcium chloride. This process of gelatinizing the starch, adding pancreatin, and dialyzing was repeated daily until the test for starch was negative. The pentosan, which is relatively insoluble in water, was recovered in a Sharples' supercentrifuge or precipitated with one volume of ethanol at 0°–2° C. The latter procedure gave higher recoveries but also a higher protein content. From 100 lb. of flour about 0.5 lb. of "squeegee" pentosan containing 60 to 75% pentosan and 1 to 3% protein was obtained.

Methods

A few of the microorganisms tested for pentosanase production were isolated from enrichment cultures, but the majority were selected from the laboratory's culture collection. The test medium consisted of: yeast extract, 5 gm.; K_2HPO_4, 2 gm.; urea, 1 gm.; NH_4Cl, 1 gm.; $MgSO_4.7H_2O$, 1.0 gm.; $ZnSO_4.7H_2O$, 0.01 gm.; $CuSO_4.5H_20$, 0.0005 gm.; $MnSO_4.H_2O$, 0.0075 gm.; $FeSO_4.7H_2O$, 0.01 gm.; $CaCO_3$, 1.0 gm.; and either water soluble pentosan or glucose, 10 gm. per liter. Inoculum for the molds was prepared by transferring spores from stock slants to 25 ml. of trypticase soy broth (B.B.L.) in 250 ml. Erlenmeyer flasks that were then incubated for 48 hr. at 30° C. on a rotatory shaker (200 r.p.m., 1¼ in. eccentricity). The mycelium was homogenized, aseptically, in a Waring Blendor, and 1 ml. of the homogenate was used to inoculate 50 ml. of test medium in 500 ml. Erlenmeyer flasks. The molds were grown for three days on the rotatory shaker.

The presence of pentosanase was detected by mixing a portion (0.2 ml.) of the suitability diluted culture filtrate with a 0.85% pentosan solution (5 ml.) buffered at pH 5.5 with 0.05 *M* acetate and the mixture incubated for 30 min. at 30° C. The viscosity was then measured in a pipette-viscometer and the enzyme activities calculated as described by Blackwood (6). Bacteria were tested for pentosanase production in the same way except that the inoculum was a 24-hr. culture in nutrient broth, the cultures were grown for two days, and the pentosan solution was buffered at pH 6.5 with 0.05 *M* phosphate.

Subsequent measurements of enzyme activities have been made with a slightly modified reaction mixture. This mixture consists of 4.0 ml. of 1.125% pentosan solution, 0.3 ml. of 0.75 *M* buffer, and 0.2 ml. of suitably diluted enzyme. The initial viscosity in the pipette-viscometer (outflow time for water: 12 sec.) described by Blackwood (6) is 12 to 13 times that of water and 10 times that of a fully hydrolyzed sample. When graded amounts of three different culture filtrates of *B. pumilus* B12 were added to this mixture and held, along with suitable controls, at 30° C. for 30 min., the percentage decline in viscosity (12) between 25 and 85% was linearly related to the logarithm of enzyme concentration (Fig. 2). A unit of pentosanase was arbitrarily defined as the amount of enzyme that will reduce by 50% the

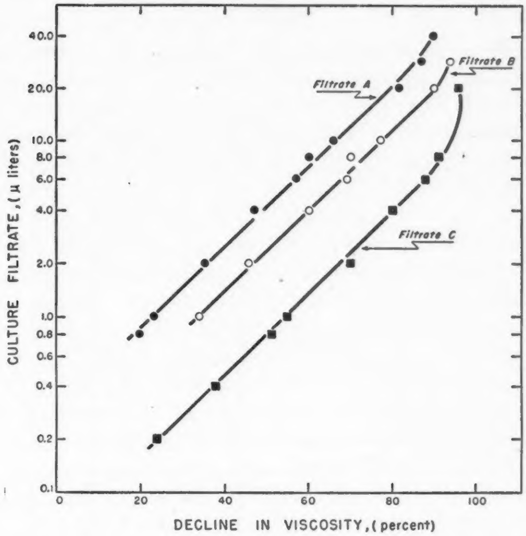

FIG. 2. The relation between decline in viscosity and the amount of enzyme.

viscosity of the standard substrate in 30 min. at 30° C. The pentosan solution was relatively stable and could be stored at 2° C. for three to four days.

Nitrogen was determined by the semimicro Kjeldahl method with a copper-selenium catalyst. The factor 5.7 was used to calculate protein. Pentosans were determined by the method of Adams and Castagne (2).

The pentosans were hydrolyzed and the sugars extracted by the procedure of Hampton, Haworth, and Hirst (10). The sugars were identified by descending paper chromatography using water saturated *n*-butanol or the ethyl acetate:acetic acid:water solvent (ratio 3:1:3). Glucose, galactose, mannose, galacturonic acid, ribose, xylose, and L-arabinose were used as controls. The position of the sugars was identified with the aniline malonate reagent of Kilgour and Dutton (11) or the silver nitrate reagent of Trevelyan, Proctor, and Harrison (22).

Experimental

Survey of Microorganisms for Production of Pentosanases

One hundred and twelve molds, 19 streptomycetes, and 129 bacteria were tested for their ability to decompose the water soluble pentosan of wheat flour. Fifty-four molds produced the required enzymes when grown on water soluble pentosan, but only six of these cultures gave active filtrates when grown on glucose, and the activity was weak (Table III). The pentosanases produced by fungi thus appear to be adaptive. The most active genera were *Alternaria*, *Aspergillus*, *Fusarium*, *Trichothecium*, and *Trichoderma*. None of the five active streptomycetes possessed a constitutive pentosanase.

TABLE III

SURVEY OF MICROORGANISMS FOR PRODUCTION OF PENTOSANASES

Genera	No. of cultures tested	No. producing pentosanases	No. with constitutive pentosanases
Alternaria	15	13	0
Ascochyta	1	0	0
Aspergillus	19	14	1?
Byssochlamys	1	0	0
Candida	2	0	0
Chaetomium	1	1	0
Claviceps	1	0	0
Fusarium	16	13	3?
Geotrichum	1	0	0
Gliocladium	2	0	0
Helminthosporium	1	1	0
Mortierella	1	0	0
Myrothecium	2	2	0
Neurospora	1	1	0
Paecilomyces	2	0	0
Penicillium	19	9	2?
Rhizopus	4	0	0
Stemphyllium	2	1	0
Thielaviopsis	1	0	0
Trichoderma	6	2	0
Trichothecium	4	4	0
Torulopsis	2	0	0
Ustilago	3	0	0
Zygorhynchus	2	0	0
Streptomyces	19	5	0
Bacillus	115	66	28
Erwinia	2	1	0
Pseudomonas	4	1	0
Unidentified bacteria	8	8	3
Unidentified mold	3	3	0

TABLE IV

SURVEY OF *Bacillus* SPP. FOR PRODUCTION OF PENTOSANASES

Species	No. of cultures tested	No. producing pentosanases	No. with constitutive pentosanases
B. alvei	9	1	1
B. brevis	4	0	0
B. cereus	7	1	0
B. circulans	2	0	0
B. coagulans	1	0	0
B. licheniformis	7	3	2
B. macerans	6	4	3
B. megaterium	5	2	0
B. para-alvei	3	0	0
B. polymyxa	17	17	6
B. pumilus	28	26	6
B. sphaericus	5	0	0
B. subtilis	21	12	10

Of the 76 bacteria that attacked the pentosan, 31 possessed constitutive pentosanases and all of these were bacilli. The most active species were *Bacillus licheniformis*, *B. polymyxa*, *B. pumilus*, and *B. subtilis* (Table IV). *B. pumilus* was of special interest because it does not produce amylases.

The extracellular pentosanases of representatives of the fungi and bacilli also hydrolyzed the pentosan of "squeegee" starch. For example, the viscosity of 5 ml. of a 0.25% suspension was reduced in 30 min. at 30° C. from 20 times that of water to 1.1 times that of water when 0.1 ml. of the culture filtrate from *B. pumilus* B12 was added.

The water soluble pentosan on hydrolysis gave glucose, galactose, xylose, and arabinose. The latter two sugars were most abundant. The "squeegee" pentosan gave glucose, xylose, and arabinose. As the molds and streptomycetes as well as some of the bacilli possessed adaptive enzymes the ability of these sugars to induce pentosanase production was determined. Only those strains that produced medium to relatively large amounts of pentosanase were included in the test. The bacterial cultures were sampled and tested for enzyme activity at 42 and 66 hr. while the molds were sampled at 48 and 72 hr. The units of pentosanase presented in Table V and Table VI are the highest found in the two samplings from duplicate runs made at different times.

TABLE V

THE EFFECT OF SUGARS ON THE PRODUCTION OF PENTOSANASES BY SOME BACTERIA

Organism	Strain No.	Units of pentosanase per ml. of culture filtrate					
		Basal	D-glucose	D-galactose	L-arabinose	D-xylose	Pentosan
B. pumilus	B12	10	5	6	4	1460	2850
	B319	25	15	17	33	1450	2900
	B387	4	9	6	4	1525	1700
B. megaterium	B477	Trace	Trace	8	Trace	7	50
B. polymyxa	B475	3	3	8	5	7	460
	B505	3	Trace	2	Trace	2	640
	B507	3	18	9	4	50	750

TABLE VI

THE EFFECT OF SUGARS ON THE PRODUCTION OF PENTOSANASES BY SOME MOLDS

Organism	Strain No.	Units of pentosanase per ml. of culture filtrate				
		D-glucose	D-galactose	L-arabinose	D-xylose	Pentosan
Alternaria sp.	99	0	0	0	5	10
Aspergillus niger	18	2	3	325	1087	2800
	23	2	0	270	1850	2600
Aspergillus fumigatus	997	Trace	0	17	770	1630
Aspergillus sp.	72	0	0	69	460	2000
Chaetomium sp.	319	0	0	87	6	1300
Fusarium sp.	30	0	7	2	52	130
	118	0	0	3	13	148
Myrothecium verrucaria	532	2	2	10	6	120
Penicillium sp.	40	2	2	35	39	1650
	94	3	3	108	179	950
Trichoderma sp.	198	0	0	140	2	800
	1113	0	0	185	255	1000
Trichothecium sp.	425	0	0	9	2	145
Streptomyces sp.	5	0	0	2	3	422
	328	0	0	23	4	438

Glucose, galactose, and arabinose did not induce the production of pentosanases by *B. pumilus* or *B. polymyxa* whereas xylose and the water soluble pentosan did (Table V). Galactose and xylose may be considered as inducing enzyme formation in *B. megaterium* but the yields are too low for an adequate test.

Glucose and galactose are similarly without effect on the molds and streptomycetes (Table VI). Arabinose, however, is more effective with the molds than the bacilli and some strains such as *Chaetomium* sp. 319 and *Trichoderma* sp. 198 produced more pentosanases on arabinose than on xylose.

Discussion

The structure of the water soluble pentosans of wheat has been studied. Ford and Peat examined a pentosan that was associated with a β-amylase preparation from wheat (8). They concluded that the basic repeating unit consisted of one galactose residue and three xylose residues joined in a straight chain to which were attached branches consisting of one xylose residue and a terminal arabofuranose residue. Perlin did not believe that galactose formed part of the main pentosan nucleus of the water soluble pentosan that he obtained from wheat flour (17). The galactose and glucose present in his preparations could be readily segregated while the molecules were still relatively large. The basic repeating unit was described as a straight chain of anhydro-D-xylose residues linked β-1, 4 to which are appended single units of anhydro-L-arabofuranose through 1, 2- and 1, 3-linkages.

Montgomery and Smith investigated the structure of the water insoluble pentosan of "squeegee" starch isolated in this laboratory (14) and found it to be closely related to the water soluble pentosan examined by Perlin (17).

The induction of pentosanase synthesis in *B. pumilus* by xylose, but not arabinose may indicate that the enzyme produced is an xylanase. Likewise the molds, since they respond as a rule to both arabinose and xylose, may produce both arabinases and xylanases. The failure of galactose to stimulate the production of enzymes that attack the water soluble pentosan strengthens the proposition that the enzymes studied here are true pentosanases.

Acknowledgments

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ON THE CHEMICAL COMPOSITION OF THE CELL WALLS OF THE ACTINOMYCETALES AND ITS RELATION TO THEIR SYSTEMATIC POSITION[1]

BY R. J. AVERY[2] AND F. BLANK

Abstract

Washed and dried cultures of *Actinomyces bovis*, *A. israeli*, *Nocardia asteroides*, *Streptomyces albus*, *S. griseus*, and *Micromonospora chalcea* were treated as described by Scholl (1908). Neither chitin nor cellulose, which form the framework of the Eumycetes, could be isolated. Therefore, from this chemical point of view the Actinomycetales have nothing in common with the true fungi, but rather with bacteria. These findings are in complete agreement with the morphological features of the Actinomycetales which indicate their place among the bacteria.

I. Introduction

The systematic position of the Actinomycetales has never been settled satisfactorily. Their relationship to other groups of microorganisms has properly been described by Vuillemin (26) who called their position "incertae sedis". A number of authors (4, 8, 12, 22, 30) place the Actinomycetales among the bacteria, others (5, 6, 16, 17, 22) among the fungi. A third group (9, 13, 27) believes that the Actinomycetales form a transition group between bacteria and fungi. However, it should be stated that in many cases the determination of the systematic position was made arbitrarily.

The chemical composition of the cell walls of fungi is not influenced qualitatively by ecological factors (1, 28). Von Wettstein (28) was the first who used this characteristic for taxonomic, systematic, and phylogenetic considerations and speculations. For the same reason, we have examined a number of species representative of four genera of the Actinomycetales in order to ascertain whether their skeleton is composed of chitin or cellulose, since these two water-insoluble polysaccharides display a very particular distribution among microorganisms (7, 18, 23, 28, 29).

II. Materials and Methods

Since the absence of chitin in *Mycobacterium tuberculosis* had been reported by Schmidt (23) representative species of the four other genera of the Actinomycetales were cultured and investigated:

Genus *Actinomyces* Harz, 1877

The species *A. bovis* Harz, 1877, and *A. israeli* (Kruse) Lachner-Sandoval, 1898, as listed in the *Sixth Edition of Bergey's Manual of Determinative*

[1] *Manuscript received June 7, 1954.*

Contribution from the Department of Bacteriology and Immunology, McGill University, Montreal. Presented as part of a paper at the Third Annual Meeting of the Canadian Society of Microbiologists, June 4-6, 1953, Guelph, Ontario.

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Bacteriology (2) were isolated from bovine mandibular lesions by one of the authors (R.J.A.). The characteristics of the two species were very similar to those given in the Manual except that both organisms are strict anaerobes and *A. israeli* has never produced aerial hyphae. The main criteria for differentiating the species were the colonial and microscopic morphologies, which agreed with those in the Manual.

A single colony of each species was picked from a blood agar plate that had been incubated for five days in a MacIntosh-Fildes jar and inoculated into 10 cc. of a beef heart infusion broth (15) to which 1.0% glucose, 0.05% agar, 0.1% sodium thioglycollate, and 0.0002% methylene blue were added. After a week the growth was transferred to an Erlenmeyer flask containing 500 cc. of this medium. The resulting growth was inoculated into a carboy containing 16 liters of the medium mentioned before. All cultures were incubated at 37° C. The cultures grown in the carboys were autoclaved after seven to eight days, at 120° C. for one hour. The growth was harvested in a DeLaval Separator and dried in an Edwards Centrifugal Freeze Drier. Ten grams of dried material were obtained from five carboys inoculated by the method described.

Genus *Nocardia* Trevisan, 1889

A culture of *N. asteroides* (Eppinger) Blanchard, 1895, was obtained through the courtesy of Prof. Johanna Westerdijk, Centraalbureau voor Schimmelcultures (C.B.S.), Baarn, Holland. The organism was cultured on Sabouraud glucose agar to which 0.001% thiamine and 0.005% inositol were added. The cultures were incubated at room temperature for five to six weeks and autoclaved at 120° C. for 20 min. The growth was collected from the still liquid medium on a Buchner funnel, washed, and dried by lyophilization.

Genus *Streptomyces* Waksman and Henrici, 1943

Cultures of *S. albus* (Rossi Doria, emend. Krainsky) Waksman and Henrici, 1943 and *S. griseus* Krainsky, 1914, were obtained through the courtesy of Prof. Johanna Westerdijk, C.B.S., Baarn, Holland.

Both organisms were cultured on the same medium and treated in the same manner as *N. asteroides*.

Genus *Micromonospora* Ørskov, 1923

A culture tentatively identified as *M. chalcea* (Foulerton) Ørskov, 1923, was obtained through the courtesy of H. L. Jensen, Statens Planteavls-Laboratorium, Lyngby, Denmark.

The organism was cultivated on LBY medium (14) to which 1.5% agar was added. The cultures were incubated at room temperature a period of 10 weeks and then autoclaved at 120° C. for 20 min. The organisms were harvested in a DeLaval Separator from the still liquid medium and dried by lyophilization.

The dried material obtained from the species mentioned before was treated as described by Scholl (1, 24) to establish the presence or absence of chitin and cellulose.

III. Results

Neither chitin nor cellulose could be isolated from any of the Actinomycetales examined. Usually, there was no solid organic material left after the first boiling in 10% potassium hydroxide. These findings are in agreement with those obtained by Rippel and Witter (19), who were unable to find chitin in five different, unidentified aerobic "Actinomycetes".

IV. Conclusions

Chitin and cellulose which form the framework of the cell walls of fungi (7, 11, 20, 21, 28) never occur together (7). To our knowledge, chitin has never been isolated from bacteria. Only a "substance similar, but not identical with chitin" was obtained by Heidelberger and Kendall (10) from pneumococci. Cellulose has been found in two bacteria only, *Acetobacter xylinum* (3) and *Sarcina ventriculi* (25). These results indicate that chitin does not occur in bacteria, although it is the most commonly occurring skeletal substance of fungi. Cellulose is a rarity in bacteria and it is less common than chitin among the fungi, where it is found only in the *Oomycetes*.

One of the conclusions drawn by von Wettstein (28) from his histochemical investigations of the cell walls was the following: "Die Einheitlichkeit im Fehlen von Chitin ist aber so gross, dass ich sogar glaube, bei strittigen Formen wie Mycobakterien könnte man die Zugehörigkeit zu Bakterien oder Pilzen auf diesem Wege zu entscheiden versuchen". From this point of view of comparative biochemistry, i.e. the chemical composition of the framework, there does not seem to be any doubt that the Actinomycetales investigated do not belong to the fungi but to the bacteria.

This characteristic, the chemical composition of the cell wall, would not carry too much weight since, in this case, it is a negative one, because both chitin and cellulose are absent in these organisms. However, our chemical findings are in complete agreement with a number of the morphological and physiological features of the Actinomycetales, such as the width of the thallus, sensitivity to acids, etc., which are characteristic for bacteria, but not for fungi. The formation of long, branched threads which is very often regarded as the most fungus-like structure of the Actinomycetales may also be observed in bacteria. It seems likely that these organisms are more closely related to certain groups of bacteria such as the Corynebacteria than to the fungi. As these facts show, the Actinomycetales have nothing in common with the Eumycetes.

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